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

Ph.D. dissertation

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2 LIST OF ABBREVIATIONS (in alphabetic order)

2,4-D - 2,4-dichlorophenoxyacetic acid
ACC - 1-aminocyclopropane-1-carboxylate
AHL – Acyl-homoserine lactone
AIP - Autoinducing polypeptides
ANOVA – Analysis of Variance (one-way)
BCA – Biocontrol agents
BLAST – Basic local alignment search tool
CAS - Chromeazurol S
CCF – cell-free culture filtrate
CFU – Colony forming unit
CVM - *Chromobacterium violaceum* Medium
DDW – Double distilled water
EPSPS - 5-enol-pyruvyl-shikimate-3-phosphate synthase
ER - Enrichment Medium
HBED - N,N'-bis (2-hydroxybenzyl) ethylenediamine-N,N'-diacetic acid
HPLC-MS – High-performance liquid chromatograph coupled to mass-spectrometer
HPLC-HRMS - High-performance liquid chromatograph-high-resolution mass spectrometry
HTA - Homoserine transacetylase
IAA – Indole-3-acetic acid
LB - Luria-Bertani
MCPA - 2-methyl-4-chlorophenoxyacetic acid
MEE - Supplemented Meat Medium
MeOH - Methanol
MM - Minimal Medium
MSM - Mineral Salt Medium
MVM - Muromtsev's Medium
MW – Molecular Weight
m/z – mass-to-charge ratio
OD – Optical density
PCR – Polymerase chain reaction
PDA - Potato Dextrose Agar
PDB - Potato Dextrose Broth
PEG 6000 - Polyethylene glycol (molar weight 6000)
PGPB – Plant growth-promoting bacteria
PGPR - Plant growth-promoting rhizobacteria
PM - Pikovskaya Medium
PPDB - Pesticide Properties DataBase (http://sitem.herts.ac.uk/aeru/ppdb/)
PW- Peptone Water
QS – Quorum-sensing
ROS – Reactive oxygen species
Rpm – Revolutions per minute
RT – Retention time
SD – Standard deviations
SMM - Solid Minimal Medium
SSM - Standard Succinic acid Medium
SZMC – Szeged Microbiological Collection
YEG - Yeast Extract Glucose

3 INTRODUCTION

3.1 Plant growth-promoting bacteria as biofertilisers in sustainable agriculture

3.1.1 Benefits of plant growth-stimulating bacteria

Several crucial functions of soil ecosystems, such as carbon, nitrogen, and phosphorus cycling, carbon storage, as well as the elimination of toxic xenobiotics (substances that are foreign to the environment, such as chemicals with industrial, household, and agricultural origin) (Patterson et al. 2010) depend remarkably on the activity of soil microbiome (Aislabie and Deslippe 2013). These processes represent significant importance in both ecological and agricultural functions, and numerous studies have investigated the role and potential application of beneficial microorganisms in agricultural systems (Timmusk et al. 2017, Gouda et al. 2018, Rouphael and Colla 2020). Certain microbes, including plant growth-promoting rhizobacteria, nitrogen-fixing cyanobacteria, biopesticides and strains with the ability to degrade different xenobiotics proved to have great potential to improve both soil quality and crop yield (Aislabie and Deslippe 2013, Etesami and Maheshwari 2018). *Acetobacter pasteurianus* AJK-7 and *Stenotrophomonas rhizophila* AJK-3 increased the nitrogen content in both the shoot and root of wheat and increased its growth *in vitro* (Majeed et al. 2015). *Pseudomonas putida* Rs-198 increased significantly the macro- and micronutrient content of tomato fruits grown in a controlled greenhouse (He et al. 2019), while *P. mendocina* improved soil aggregate stability together with dehydrogenase and phosphatase activities *in vivo* (Kohler et al. 2006). Beneficial microbial strains are of great importance from both agricultural and ecological points of view. The extensive use of synthetic pesticides and fertilisers led to soil contamination and raised subsequent risks for the environment and human health as well. Many pesticides, including the globally used herbicide glyphosate, were reported to have adverse effects on the beneficial soil microbiome, bees, fish, and mammals (Rahman et al. 2018, Gouda et al. 2018, Van Bruggen et al. 2018). Heavy metals might also be accumulated in agricultural soils and raise serious concerns for ecosystems and humans (Jaiswal et al. 2018). Human pressure on soils is reaching critical limits, and thoughtful soil management is vital to preserve the ecosystems and biodiversity (FAO, Main Report, 2015). Therefore, microbe-based biofertilisers and biocontrol strains are considered to be effective, environment-friendly, and suitable alternatives to hazardous chemical pesticides (Gouda et al. 2018, Basu et al. 2021). Numerous microbial products have been developed and already applied in agriculture to enhance crop productivity and protect plants from pathogens (Table 1).

Table 1. Examples of the commercialised microbial bioproducts available for plant growth promotion and crop protection.

Product	Country	Active strain or microbial metabolite	Target crop; influence	Reference*
Nodulator® PRO	Canada	*Bradyrhizobium japonicum*, *Bacillus amyloliquefaciens*	Soybean; nitrogen fixation (*B. japonicum*), protecting seedlings from diseases caused by *Rhizoctonia* and *Fusarium* spp. (*B. amyloliquefaciens*)	https://agriculture.basf.ca/west/products/solutions/nodulator-pro-100.html
Nodulator® XL LQ		*Rhizobium leguminosarum* bv. *viceae* (strain 1435)	Peas and lentils; nitrogen fixation	https://agriculture.basf.ca/west/products/solutions/nodulator-xl-lq.html
Cedomon®	Sweden	*P. chlororaphis*	Biocontrol of leaf spots in barley	https://www.lantmannenbioagri.com/biological-seed-treatment/
Cerall®			Biocontrol of common wheat bunt	
BactoFil® Pillangós	Hungary	*R. leguminosarum*	Peas; nitrogen fixation	https://agrobio.hu/hu/termekek-kijuttatas/talajolto-termekek/bactofil-pillangos/
BactoFil ® Kalászos		*Azotobacter vinelandii*, *Azospirillum brasilense*	Cereal crops; nitrogen fixation and phytohormone production (auxins, gibberellins)	https://agrobio.hu/hu/termekek-kijuttatas/talajolto-termekek/bactofil-kalaszos/
CONSENSUS®	USA	Chitosan, salicylic acid, and indole-3-butyric acid	Different crops; plant growth regulation	https://www.lovelandproducts.com/product/consensus%C2%AE
Accomplish LM	USA	*B. pumilus*, *B. megaterium*, *B. licheniformis*	Different crops; improvement of nutrient availability, plant performance, reduction of salt stress	https://www.lovelandproducts.com/product/nitrogen-management-accomplish-lm

*Accessed on December, 19th, 2021

In general, agriculturally beneficial bacterial inoculants can be categorised as (1) plant growth-promoting bacteria/plant growth-promoting rhizobacteria (PGPB/PGPR, biofertilisers), including rhizospheric and non-rhizospheric bacteria, which enhance crop growth and development, as well as (2) biocontrol agents (BCAs/biopesticides), playing role in crop protection by controlling different plant pathogenic organisms (Bashan and Holguin 1998; Vessey 2003, Calvo et al. 2014,). As the benefits of PGPB and BCA have been proven both by *in vitro* and *in vivo* studies, the global market of these agents is growing continuously (Bashan et al. 2014, Calvo et al. 2014, Timmusk et al. 2017, Arthurs and Dara 2018, Gouda et al. 2018, Rahman et al. 2018). It is estimated that the global market of biofertilisers might reach 3.5 billion USD by 2025 (Basu et al. 2021). The current studies include the isolation and identification of novel species with the potential of agricultural application, the investigation into the various mechanisms involved in plant growth promotion and protection, the molecular and eco-physiological characterisation of the promising microbial agents, the evaluation of their tolerance to different abiotic and biotic stress factors and the development of new microbial products, consisting of sole microorganisms or consortia of different microbial species providing multiple beneficial effect (Etesami and Maheshwari 2018; Basu et al. 2021; Fiodor et al. 2021). In the roadmap of commercialisation, the initial step after strain isolation usually covers the investigation of the possible plant growth-promoting characteristics of the isolates, which can influence crop growth in direct or indirect ways (Timmusk et al. 2017, Basu et al. 2021)

3.1.2 Direct and indirect mechanisms of plant growth enhancement of bacteria

Beneficial microorganisms can promote crop growth by different direct and indirect mechanisms. Three basic essential macronutrients for all plants, nitrogen (N), phosphorus (P), and potassium (K) can be provided by PGPB through N-fixation (Kuypers et al. 2018), as well as the solubilisation of inorganic insoluble P (Sharma et al. 2013) and K-sources (Etesami et al. 2017). Moreover, PGPB synthesize different phytohormones and other molecules such as indole-3-acetic acid (IAA), cytokinins, gibberellins and siderophores (Pahari et al. 2017, Vishwakarma et al. 2020). Phosphorus plays an important role in plant metabolism, including key processes, such as photosynthesis, energy transfer, and sugar biosynthesis (Khan et al. 2010a). However, as P in soils is presented mostly in forms that are unavailable for plants, this element is considered to be the main crop yield limiting factor (Sharma et al. 2013). Therefore, soil microorganisms capable of solubilising inorganic P compounds are considered as plant growth-promoting agents as they provide plants with solubilised P. The phytohormone IAA plays an important role in plant growth and development (Duca et al. 2014, Keswani et al. 2020). IAA takes part in many vital processes, such as cell elongation, division, and differentiation (Luo et al. 2018, Majda and Robert 2018). Therefore, IAA-synthesising strains are attractive as potential plant growth-promoting agents. Ammonia (NH_3) produced by microorganisms is of high importance for plant development (Kayasth et al. 2014) and the produced NH_3 might be further used by plants as N-source (Patil et al. 2016).

Importantly, biofertilisers are recognized as effective agents to help plants sustain abiotic stress factors abundant in agricultural fields (Etesami and Maheshwari 2018, Fiodor et al. 2021). Direct and indirect mechanisms involved in plant growth promotion and alleviated stress factors can be seen in Figure 1 (Etesami and Maheshwari 2018).

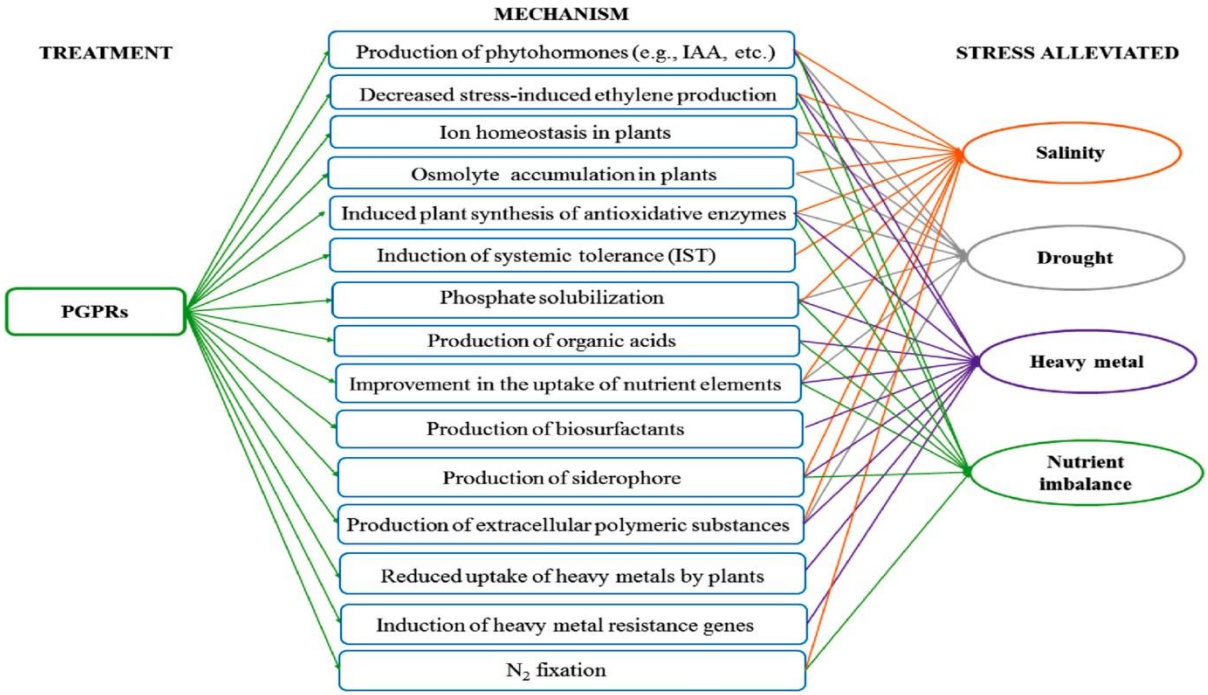


Figure 1. Different mechanisms applied by beneficial microorganisms to mitigate abiotic stress factors in plants (Etesami and Maheshwari 2018)

Abiotic stress factors are the most uncontrollable factors, therefore, agents capable of enhancing the tolerance of plants have raised the interest of both farmers and researchers (Etesami and Maheshwari 2018, Basu et al. 2021, Fiodor et al. 2021). Several mechanisms are involved in providing plants with the potential to resist abiotic stress factors (Figure 1), in which the production of IAA and siderophores are of particular importance, as they may mitigate stress by having a positive influence on cell elongation and differentiation, as well as on iron suppliment under iron-depleted circumstances.

3.1.3 The role of auxin-type phytohormones of bacterial origin in plant growth and development

Plant hormones are important during the entire life cycle of plants. Currently, seven types of phytohormones (auxins, gibberellins, cytokinins, brassinosteroids, abscisic acid, ethylene, and jasmonic acid) are known to influence plant growth and development via direct and indirect modes of action (Tanimoto 2005). Among phytohormones, auxin-type compounds are of great importance as they are involved in controlling nearly all aspects of plant growth (Duca et al. 2014). Auxins represent a heterogeneous group of carboxylic acid signaling molecules involved in the regulation of different physiological aspects of plants (Wagi and Ahmed 2019). Auxins attract scientific attention not only because of their capacity to mediate plant growth but also because the physiological processes governed by auxins are crucial in the structure and functions of plants (Teale et al. 2006). The most common natural auxin –IAA is the first phytohormone identified and recognized as a crucial plant hormone in various processes (Teale et al. 2006, Duca et al. 2014, Keswani et al. 2020). Chemically, IAA is a substance in which carboxylic acid is connected via methylene group to the C3 position of an indole ring, a colourless solid compound, soluble in polar organic solvents (Keswani et al. 2020). It initiates the growth of roots, leaves, and flowers, and plays an important role in cell division and elongation (Duca et al. 2014, Keswani et al. 2020).

The growth promotion by microbial auxins is one of the most important direct stimulating mechanisms of bacteria in plant growth (Wagi and Ahmed 2019). IAA, the

phytohormone possessing the highest physiological activity, can be produced by a broad range of soil microorganisms (Shrivastava and Kumar 2011, Keswani et al. 2020). Various strains, such as *Aureobasidium* sp. BSS6, *B. subtilis* Mt3b, *Ensifer adhaerens* TMX-23, and *P. resinovorans* have been reported as IAA-producing strains with the ability to promote plant growth (Zhou et al. 2013, Khan et al. 2016, Habibi et al. 2019, Wagi and Ahmed 2019). The most common metabolic pathway utilised by bacteria to produce IAA involves the amino acid tryptophan and its intermediates, although recently the tryptophan-independent mechanism of IAA synthesis has also been reported (Keswani et al. 2020).

Numerous studies have investigated the potential of microbial species to produce IAA in agricultural applications. IAA-producing *Preussia* sp. BSL10 isolated from *Boswellia sacra* tree and further applied to the saplings of the host, could significantly improve plant growth (Khan et al. 2016), while the highest onion yields were observed following treatment with *A. chroococcum* and *B. subtilis*, individually, both capable of IAA synthesis (Čolo et al. 2013). Moreover, phytohormones, including auxins, gibberellic acid, cytokinins, and abscisic acid could not only improve crop growth and development, but were also able to attenuate the influence of abiotic stress factors on crops via the mediation of different stress responses, such as the regulation of growth and development, or nutrient allocation (Duca et al. 2014, Etesami and Maheshwari 2018). The inoculation of tomato with *P. chlororaphis* ssp. *aureofaciens* M71 resulted in elevated IAA production and antioxidant activity under water stress conditions, and increased expression of genes involved in the biosynthesis of leaf terpenes (Brilli et al. 2019) and treating the host *Sedum alfredii* with IAA-producing *P. fluorescens* Sasm05 promoted plant growth under Cd stress (Chen et al. 2017).

In addition to the traditional view that bacteria promote plant growth via the synthesis and supplementation of crops with IAA directly, the hypothesis of the potential role of bacteria in plant growth by IAA regulation was suggested by Duca et al. (2014). It was emphasized that IAA at high concentrations might has a detrimental effect on plant growth, but as IAA is important in bacterial metabolism as well, the degradation of the plant-derived IAA by bacteria, when it reaches detrimental concentration, could also help to overcome this side effect.

Therefore, as IAA is the best known phytohormone directly involved in various important physiological processes of crop growth, IAA-synthesising microbial strains are attractive as potential plant growth-promoting agents.

3.1.4 Bacterial siderophores as plant growth-promoting agents

Siderophores are low-molecular-weight, iron-specific (FeIII) compounds released by microorganisms under iron-limited stress conditions. Siderophores can solubilise, bind and transport iron into the bacterial cells through specific membrane receptors (Schwyn and Neilands 1987, Payne 1994). In medicine, the ability to produce siderophores is a virulence factor as the siderophore production by pathogens was often detected during infections. Siderophores were identified in fungal pathogens such as *Histoplasma capsulatum* and *Candida albicans*, as well as the bacterial species *Salmonella* sp., *Vibrio* sp. and *P. aeruginosa* (Payne 1993, Cunrath et al. 2020). However, recent studies have reported the potential application of bacterial siderophores in various fields. The addition of siderophores promoted the growth of previously uncultivable bacteria *in vitro*, while the Trojan horse strategy, in which antimicrobial compounds are conjugated with siderophores and transported inside the microbial cells, was studied as a way to overcome the difficulties of drug delivery due to membrane impermeability (Saha et al. 2016). In agriculture, bacteria with the potential of siderophore production are known as beneficial microorganisms providing plants with multiple functions. Besides supplying plants with soluble iron (Sharma and Johri 2003, Saha et al. 2016), the production of siderophores enables PGPB to compete with plant pathogens

and provide better host protection (Pahari et al. 2017). The detailed mechanisms of siderophore-mediated suppression of plant pathogens are described in Subsection 3.2.4. Another way for the utilisation of siderophores is bioremediation. Siderophores were reported to play role in the detoxification of substrates polluted by heavy metals (Cr, Al, Cu, Pb, Zn, etc.) due to their metal-chelating abilities (Saha et al. 2016).

Altogether, the characteristics of bacterial siderophores, such as supplying crops with iron, protection from various pathogens, and potential of cleaning heavy metal-polluted agricultural soils up make siderophore-producing microorganisms promising candidates for plant growth promotion and crop protection.

3.2 Microbial biocontrol of plant pathogens

3.2.1 Losses of crop yield due to plant pathogens

Farmers have been struggling with controlling organisms harming crop products during the entire history of agricultural practice, which started around 10 000 years ago (Oerke 2006). The term "pest" is used to define living organisms that have adverse effect on food production and consumption, including approximately 67,000 different species of weeds, plant pathogenic microbes and different animals that are harmful for both agricultural plants and animals (Oerke et al. 1994, Chandler et al. 2011).

The adverse effects of pests on the food production might be divided into 2 groups: quantitative and qualitative. Reduced crop yields are regarded as quantitative damage, while qualitative losses include decreased market quality, limited shelf file, and product contamination, e.g., due to mycotoxin production (Oerke 2006). Furthermore, crop reduction due to pests is also differentiated as potential and actual losses: potential losses include the total capacity of pests to damage a plant in the absence of crop protection measures, while actual losses mean crop damage appearing even along with the application of pest management strategies (Oerke and Dehne 2004).

Among pests, the rate of potential losses caused by weeds is the highest (32%), followed by animal pests (18%) and microbial pathogens (15%). However, the efficacy of controlling microbial pathogens and animal pests using mainly pesticides is remarkably lower than the control of weeds using both chemical and mechanical methods, accounting for 32, 39, and 68%, respectively (Oerke and Dehne 2004). In economic equivalent, the estimated crop losses caused by plant diseases costs approximately 40 billion USD per year (Rahman et al. 2018). For example, wheat rusts and *Fusarium* head blight alone caused yield losses of 5 and 3 billion USD per year, respectively (Schumann and D'Arcy 200, Savary et al. 2012).

Agrochemicals or synthetic pesticides are vital elements of the modern pest management aiming the prevention of potential crop losses (Rahman et al. 2018). The global agrochemical market was reported to be 37.9 billion USD in 2009 (Glare et al. 2012). The losses of yield of the main crops caused by pests are estimated to be 25-40% with crop protection including the use of pesticides, while without pest control, these values may increase up to 50-80% (Oerke and Dehne 2004). However, a continuous growth of pesticide use (Carvalho 2006) resulted in the development of fungicide (Rahman et al. 2018) and herbicide (Hicks et al. 2018) resistance of the pathogens and pests, while the application of higher dosage to overcome potential resistance led to environmental pollution and human health hazard (Carvalho 2006, Thakore 2006). Certain pesticides are highly persistent and can remain in the environment for a long time. For example, due to their considerable toxicity and hazards, the organochlorine pesticides DDT and dieldrin were banned in the USA in 1970s. However, after a 30-year period of application, certain persistent organic pollutants, including dieldrin, DDT and its metabolites (DDE) could be detected in all types of food at certain levels (Schafer and Kegley 2002). Therefore, alternative, ecologically safe and

effective approaches attracted public and scientific interest both for the control of agricultural pests and the prevention of the contamination of food, soil, and ecosystems. Consequently, it resulted in the development of various alternative means of pest management, including the use of biopesticides, by which effective pest control can be achieved in an environment-friendly manner (Mnif and Ghribi 2015, Rahman et al. 2018).

3.2.2 Potential of biocontrol strains in pest management

In 1972, the first *B. thuringiensis*-based insecticide was introduced for the control of lepidopterous pests (Oerke 2006). Since that time, biological methods based on the use of beneficial microorganisms, generally called biopesticides, have been accepted as eco-friendly and low-cost approach against harmful pests (Glare et al. 2012, Mnif and Ghribi 2015, Rahman et al. 2018). In general, the term "biopesticide" refers to commercialised living organisms (plants, nematodes, and microorganisms, such as bacteria, fungi, and protozoa) or active metabolites derived from them used for the control of crop pests (Thakore 2006, Glare et al. 2012). Based on the active substance, biopesticides might be classified in 3 groups as microbial biopesticides (living microorganisms), biochemicals (active metabolites), and semiochemicals (chemical signals, e. g. insect sex pheromones) (Chandler et al. 2011). Several results have proved the effective control of various pests using either living organisms or their metabolites, 37,705 publications were found in the PubMed database for the keyword "biopesticides" in January 2022 (https://pubmed.ncbi.nlm.nih.gov/?term=biopesticides).

Bacillus and *Pseudomonas*-based biopesticides are the most proven biocontrol agents. The genus *Pseudomonas* involves Gram-negative, motile, aerobic bacteria, representing the most heterogeneous and ecologically significant group among all known bacterial species (Mnif and Ghribi 2015). Some species, such as *P. syringae* or *P. aeruginosa* are serious plant (Xin et al. 2018) or human (Pang et al. 2019) pathogens, while other species are well-recognized agents for plant growth promotion, biocontrol, and bioremediation (Mnif and Ghribi 2015). The extract of the culture supernatant of *P. chlororaphis* ssp. *aureofaciens* DSM 6698 fully inhibited the growth of *R. solani*, *Pythium ultimum*, and *Fusarium oxysporum* at 20 μg/ml concentration (Mezaache-Aichour et al. 2013). The 2,4-diacetylphloroglucinol-producing *P. putida* and *P. aeruginosa* strains exhibited antagonistic activity against *Pyricularia grisea, Gaeumannomyces graminis, F. oxysporum, Xanthomonas campestris*, and *Erwinia persicina* (Sekar and Prabavathy 2014). The volatile organic compounds produced by *P. fluorescens* B-4117 and Q8r1-96 inhibited the growth of *Agrobacterium tumefaciens* and *A vitis in vitro* (Dandurishvili et al. 2010).

In addition to the biocontrol efficacy in an environment-friendly manner, many biocontrol strains were reported to have beneficial traits to improve soil quality and plant growth, enhancing the applicability of biopesticides compared to synthetic agrochemicals. *Trichoderma* species can improve the absorption of soil nutrients by plants (Harman 2011). Weselowski et al. (2016) reported *Paenibacillus polymyxa* CR1 as a beneficial strain not only for pathogen control, but also for enhancement of plant growth and decomposition of plant residues. Moreover, according to experimental and modelling assays, biocontrol agents with different modes of action might be utilised together (Xu et al. 2011), or biopesticides might be combined with conventional chemical pesticides (Peng et al. 2014) to achieve better efficacy of pest control.

From economic point of view, currently approximately 140,000 chemicals have to be screened for the development of a potential pesticide, which requires 250 million USD and 10 years, while the commercialisation of a biopesticide costs 3-5 million USD and takes only 3 years (Glare et al. 2012). The global biopesticide market is showing an increasing tendency,

which was 672 million in 2000 (Thakore 2005), while the global sales reached 1.6 billion USD in 2009 (Glare et al. 2012).

Overall, the effectiveness of pathogen control, eco-friendly mode of action, lower costs of development, additional characteristics to improve plant growth and soil quality have raised the governmental, public and industrial interests, which can be expected to expand the prospective of biopesticides for a greener tomorrow (Chandler et al. 2011, Glare et al. 2012, Mnif and Ghribi 2015, Rahman et al. 2018).

3.2.3 Biological control of *A. tumefaciens* as a plant pathogen

The genus *Agrobacterium* consists of Gram-negative bacteria with the ability of horizontal gene transfer genes with their host plants (Subramoni et al. 2014). Agrobacteria are common inhabitants of soils, associated with roots, tubers and underground steams of plants (Matthysse 2005). Among the different species, *A. tumefaciens* and *A. vitis* are the most recognized species known to cause crown-gall formation in plants. The taxonomy of *A. tumefaciens* is discussed in many publications (Matthysse 2005, Filo et al. 2013). Based on the comparative analysis of the 16S rRNA gene sequences, the species *A. tumefaciens* has been reclassified as *Rhizobium radiobacter* by Young et al. (2001), combining both pathogenic and nonpathogenic strains. For the convenient distinction, the approach proposed by Li et al. (2019) will be applied throughout the text, in which the pathogenic strains will referred to as *A. tumefaciens*, while the nonpathogenic isolates will be mentioned as *R. radiobacter*.

A. tumefaciens possesses a serious challenge in pest management as it can infect 93 families, 331 genera, and 643 species, including both monocotyledonous and dicotyledonous plants (Conner and Dommisse 1992, Xie et al. 2021). Currently, farmers apply chemical and physical methods to control crown-gall disease. In addition to the prevention measures (selection of pathogen-free seedling material, graft union protection, renewal of trunk) and chemical methods (fumigation and solarization), Filo et al. (2013) proposed biological control as the most efficient technique to control crown-gall disease. Interestingly, among other various bacterial species, non-pathogenic *A. tumefaciens*, *A. radiobacter*, and *A. vitis* strains can also be used to control pathogenic *Agrobacterium* species (Filo et al. 2013). Furthermore, a variety of microbial species, such as *P. fluorescens, B. amyloliquefaciens, B. subtilis, Phomopsis liquidambari* were also reported as effective strains for the biological control of *A. tumefaciens* (Table 2).

Table 2. Biocontrol potential of different microbial species and their metabolites towards *A. tumefaciens* and other plant pathogenic *Agrobacterium* species

Active strain	**Antagonized strain**	**Findings**	**Reference**
P. fluorescens B-4117, *P. fluorescens* Q8r1-96	*A. tumefaciens*, *A. vitis*	Volatile organic compounds inhibited the growth *in vitro.*	Dandurishvili et al. 2010
B. amyloliquefaciens ssp. *plantarum* 32a	*A. tumefaciens* C58 and B6	Treatments of tomato plants with suspensions of both vegetative cells and spores resulted in a 79.1–87.5% reduction of the disease.	Abdallah et al. 2018a
B. amyloliquefaciens 32a	*A. tumefaciens* C58 and B6	The mixture of lipopeptides produced by the strain inhibited the tumor formation on plants and reduced the formation of biofilms by the pathogenic antagonistic strain.	Abdallah et al. 2018b
B. amyloliquefaciens 32a	*Agrobacterium* strains	Surfactin, iturin, and fengycin lipopeptides had strong protective effect.	Abdallah et al. 2015
B. methylotrophicus 39b	*A. tumefaciens* C58 and B6	Lipopeptides (surfactins, iturins (mycosubtilin), fengycins, bacillomycin D) were the main compounds that confer the biocontrol ability.	Frikha-Gargouri et al. 2016
P. fluorescens P3, *Bacillus* sp. A24	*A. tumefaciens* A334	Protection of tomato plants primarily due to the degradation of N-acyl-homoserine lactones.	Molina et al. 2003
B. velezensis CLA178	*A. tumefaciens* C58	Macrolactins (a group of macrolide antibiotics) were found to have antagonistic activity.	Chen et al. 2021
B. subtilis 14B	*A. tumefaciens*	*B. subtilis* 14B and its bacteriocins are efficient in reducing the percentage of infections in plants.	Hammami et al. 2009
B. megaterium L2	*A. tumefaciens* T-37	Phenylacetic acid and behenic acid inhibited the growth.	Xie et al. 2021
Nonpathogenic *A. radiobacter* K84	*A. tumefaciens*	The antibiotic-like bacteriocin agrocin 84 prevents the disease.	Reader et al. 2005
A. rhizogenes (*A. radiobacter*) K84	*Agrobacterium* sp.	Agrocin 434 (a hydroxamate siderophore) was reported to have the inhibitory effect on pathogenic *Agrobacterium* strains.	Penyalver et al. 2001
Rahnella aquatilis HX2	*A. vitis*, *A. tumefaciens*, *A. rhizogenes*	Suppression of sunflower crown gall disease	Li et al. 2014
Simplicillium lamellicola BCP	*A. tumefaciens*	*A. tumefaciens* was the most sensitive to mannosyl lipids.	Dang et al. 2014
P. liquidambari	*A. tumefaciens*	1-(4-amino-2-hydroxyphenyl)ethanone (AHPE) can serve as a potential pesticide.	Zhou et al. 2020

As listed in Table 2, a variety of bacterial strains were reported as effective biocontrol agents against *A. tumefaciens*. However, searching novel species with the antagonistic activity might result in new inhibition mechanisms and active substances, which can be successfully applied to prevent plant diseases and protect agricultural crops.

3.2.4 Siderophore-mediated suppression of plant pathogens and the role of extracellular enzymes and carbon sources in the biocontrol

In agriculture, bacterial siderophores are well-known not only as plant growth-promoting compounds providig iron for crops (Subsection 3.1.4), but also as promising biocontrol agents (O'Sullivan and O'Gara 1992, Saha et al. 2016). The inhibitory effect of siderophores on various bacterial and fungal plant pathogens has been widely studied (O'Sullivan and O'Gara 1992, Ambrosi et al. 2000, Mishra and Arora 2012, Sulochana et al. 2014, Michavila et al. 2017, Tao et al. 2020). One of the first detailed characterisation of siderophore-mediated suppression of bacterial growth was reported by O'Sullivan and O'Gara (1992). The main mechanism involved in this process is the competition for the limited available iron between the plant pathogens and the beneficial agents, in which production of siderophores by the latter caused iron-depleted circumstances for the plant pathogen (Figure 2) (Saha et al. 2016).

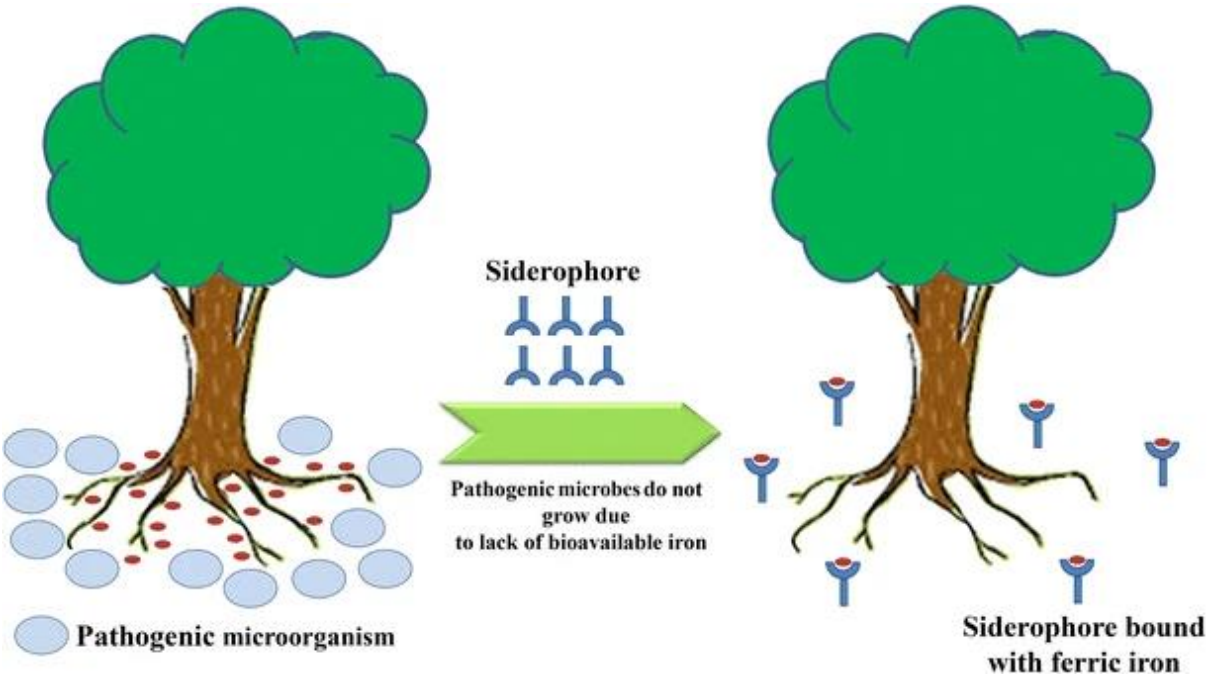


Figure 2. Siderophore-mediated suppression of deleterious organisms (Saha et al. 2016)

The siderophore-mediated suppression of plant pathogens was initially suggested in the 1970's, while the first documented evidence of this mechanism was provided by the studies of Kloepper et al. (1980). Strains B10, A1, BK1, and TL3B1 belonging to the *P. fluorescens-putida* group were found to produce fluorescent siderophores, which could inhibit the potato pathogenic *E. carotovora in vitro*. However, when the plates were amended with 1-10 µM $FeCl_3$, the strains in iron-repleted conditions did not produce siderophores, and consequently, no inhibition was observed. These findings are in accordance with the results of Mishra and Arora (2012), in which *P. aeruginosa* KA19 produced an extracellular siderophore with inhibitory effect against *X. campestris* pv. *campestris* in iron-limited conditions, but without

significant inhibition under iron-rich circumstances. The inhibition of microbial growth due to iron chelators was also proven recently by Mettrick et al. (2020). The synthetic hexadentate iron chelator N,N'-bis (2-hydroxybenzyl) ethylenediamine-N,N'-diacetic acid (HBED) remarkably inhibited both the growth and biofilm formation of clinical strains of biofilm-dwelling *P. aeruginosa* alone, while using HBED in combination with the polymyxin antibiotic colistin resulted in the nearly complete removal of the biofilm. Interestingly, Adler et al. (2012) and Michavila et al. (2017) declared that the pyochelin-type siderophore toxicity for *X. citri* ssp. *citri* was not the result of iron limitation, but due to the generation of reactive oxygen species (ROS) and lipid peroxidation. The addition of 1 mM ascorbic acid eliminated the inhibitory effect caused by pyochelin-type siderophores produced by *P. aeruginosa* and *P. protegens*. Therefore, another unknown role of siderophores in the biocontrol activity might have been discovered.

The more than 500 reported siderophores produced by various fungi and bacteria can be categorised in 3 groups based on the oxygen ligands for Fe (III) coordination: hydroxamates, catecholates, and carboxylates (Saha et al. 2016). The type of siderophores is important in terms of the biocontrol activity, as siderophore-mediated suppression high depends on types of siderophores produced by the biocontrol agent and the target pathogen. The type of siderophores released by the biocontrol agent should be different from those of the pathogen, otherwise the pathogen might exploit the siderophores of the biocontrol strain. The catecholate-type siderophore pyochelin produced by *P. aeruginosa* was effective only against bacteria with no ability to produce catecholate siderophores, while catecholate siderophore-producing bacteria were resistant to pyochelin (Adler et al. 2012). Furthermore, *A. tumefaciens* was able to promote its own growth by scavenging iron from catecholate-type siderophores produced by *A. vinelandii* to solubilise iron from insoluble iron salts (Page and Dale 1986). Typically, *A. tumefaciens* was reported as catecholate siderophore-producing species with its specific siderophore agrobactin (Leong and Neilands 1981, 1982; Sonoda et al. 2002). The agrocin 434 siderophore belonging to the hydroxamate-type produced by *A. rhizogenes* (*A. radiobacter*) strain K84 was reported to have inhibitory effect on the *A. tumefaciens* NT1 strain (Penyalver et al. 2001).

Besides, supplying plants with additional iron source, siderophore-producing strains are of high importance to limit the growth of various bacterial and fungal pathogens via siderophore-mediated suppression. Hydroxamate and carboxylate-type siderophore-producing species might be considered as promising agents for the biocontrol of *A. tumefaciens*.

Microbial enzymes were also reported to play role in the suppression of plant pathogens, and also indirectly stimulate plant growth (Mishra et al. 2020). Among enzymes, proteases, cellulases, β-1,3-glucanases, chitinases, and lipases are considered to be the most essential enzymes for the biocontrol agents, as they can be applied by the biocontrol strains directly against fungal pathogens, or in the competition for nutrients and the space in the rhizosphere (Vörös et al. 2019). *B. amyloliquefaciens* SZMC 6161J and SZMC 6225J, *B. mojavensis* SZMC 6168J, and *B. subtilis* SZMC 6179J, showing antagonistic potential towards various fungal and bacterial plant pathogens, also secreted extracellular hydrolytic enzymes, including chymotrypsin- and trypsin-like proteases, as well as lipases (Vágvölgyi et al. 2013). In the studies of Kim et al. (2014), both extracellular chitinases and the antibiotic chromobactomycin were shown to be involved in the biocontrol activity of *Chromobacterium* sp. C61against phytopathogenic fungal and bacterial species.

Nutrients, especially carbon sources play an important role in the biocontrol activity of microbes. In the studies of Sun et al. (2015), multiple strains of *Streptomyces* sp. showing less competition for carbon sources possessed improved potential to control potato scab disease. Testing of the influence of carbon sources on the antibiotic production of *P. fluorescens* CHA0 revealed that the synthesis of the antibiotics 2,4-diacetylphloroglucinol,

pyoluteorin, and pyoluteorin were promoted by glucose, glycerol, as well as fructose and manntiol, respectively. Furthermore, the production of the siderophore pyochelin and its precursor salicylic acid were stimulated by fructose, mannitol, and glucose, as well as glycerol and glucose, accordingly (Duffy and Défago 1999). The metabolism and the synthesis of the antifungal glycolipid flocculosin produced by the fungus *Pseudozyma flocculosa* was directly influenced by carbon availability and nitrogen source (Hammami et al. 2008).

Altogether, these findings propose siderophore-mediated suppression as an effective way to control plant pathogens and highlight the role of enzymes and carbon sources in the biocontrol activity of different microorganisms.

3.2.5 Metabolites produced by *P. resinovorans* as a potential agent to interfere with the quorum-sensing of *A. tumefaciens*

Bacterial cell-cell communication known as quorum-sensing (QS) involves specific chemical signal molecules (autoinducers) with the ability to freely penetrate through the cell membrane and influence the expression of certain genes. Most QS systems are species-specific, however, different autoinducers are applied by Gram-negative (acyl-homoserine lactones - AHLs) and Gram-positive (autoinducing polypeptides - AIPs) bacteria (Taga and Bassler 2003). Bacterial species use these molecules to coordinate their population behaviours. QS-systems play an important role in many cellular functions, including virulence, biofilm formation, plasmid conjugation, and antibiotic production. Therefore, compounds able to degrade, modify, or interfere with AHLs are recognized as potential novel agents in medicine and agriculture to control human and plant pathogens (Taga and Bassler 2003, Billot et al. 2020). AHLs, with fatty acid side chains ≥12 carbon atoms secreted by *P. aeruginosa* inhibited the hemolytic activity of the human pathogenic *Streptococcus pyogenes* M6 S165 strain, while 100-250 μM of two types of AHLs (oxo-C14-HSL and Oxo-C12-HS, respectively) resulted in the inhibition of its growth (Saroj et al. 2017). Pascale et al. (2011) reported β-lactones as inhibitors of homoserine transacetylase (HTA), which is involved in the biosynthesis of methionine in several fungi, Gram-positive and certain Gram-negative bacteria. Methionine plays an important role in protein translation, moreover, it is the precursor for cysteine, carnitine, taurine, and lecithin, or can be converted to *S*-adenosyl-L-methionine, which serves as a donor of methyl group in essential cell functions (Deobald et al. 2020). Therefore, Pascale et al. (2011) have proposed β-lactone inhibitors as excellent biochemical inhibitors of HTA and may potentially open the way for the development of new antimicrobial agents. Molina et al. (2003) concluded that AHL degradation was the main mode of action involved in the protection of tomato plants infected with *A. tumefaciens* by both examined AHL-producing bacterial strains (*Bacillus* sp. A24: natural AHL-producing isolate, and *P. fluorescens* P3/pME6863: transformed with the genes involved in the AHL-biosynthesis of *Bacillus* sp. A24), while no inhibition of *A. tumefaciens* was found in tomato treated with the parental *P. fluorescens* strain, which is naturally unable to produce AHLs. These findings are in accordance with the results of Uroz and Heinonsalo (2008), who observed no synthesis of QS-inhibitors or activators by *A. tumefaciens* in the presence of the culture supernatant obtained from 3 fungal isolates with the potential to degrade AHLs.

Therefore, compounds with the ability to interfere with AHLs might be considered as potential agents to control pest population via silencing their QS-activities.

3.3 Demand for the development of successful implementations in vivo

3.3.1 Current trends of using biofertilisers and biopesticides in agriculture and abiotic factors limiting their efficacy

The beneficial characteristics of microbial species applied as biofertilisers or biopesticides (described in Sections 3.1 and 3.2, respectively) and the environment-friendly manner of their action make them attractive for a large-scale application in agriculture. However, several obstacles need to be considered to achieve the successful implementation of microbial products. Based on several reviews that discussed this issue in detail (Glare et al. 2012, Timmusk et al. 2017, Basu et al. 2021), the obstacles can be divided in several categories:

1) Biological constraints include the proper selection of non-pathogenic strains that possess various growth-promoting and/or biocontrol potential are able to positively influence several crops, and can survive in a wide range of agro-ecological regions;
2) Technical obstacles represent general problems due to the nature of bioproducts. Being living organisms, microbial agents require additional precaution measures, equipment, and certain infrastructure necessary from the initial steps of the development till the strains can be applied *in vivo* at a large scale;
3) Challenges with product registration and marketing, such as the long and complicated procedure for the registration of the product and obtaining patents. Moreover, no standardised registration of microbial agents has been developed so far, therefore, the requirements vary between countries which make obstacles in worldwide trades and selling;
4) Quality and efficacy of the products in natural agricultural conditions. Strains showing beneficial characteristics *in vitro* and under greenhouse conditions might be inhibited by abiotic and biotic stress factors, and their activity can be limited *in vivo*.

Therefore, several criteria have been set to overcome the above-shown obstacles during the development of microbial products. For example, Basu et al. (2021) proposed the characteristics of an ideal microbial product, including:

- plant growth-promoting potential
- colonization of plant roots
- survival under the highly competitive conditions of the rhizosphere
- harmless to ecosystems and humans
- tolerance to physicochemical factors
- additional beneficial features (such as enhancement of soil fertility, stimulation of seed germination, bioremediation of pollutants)

Therefore, for the successful application of strains with the potential of plant growth stimulation and protection their careful eco-physiological characterisation is required to identify the optimal ecological conditions for their application, as certain abiotic factors can remarkably limit their activity *in vivo* (Basu et al. 2021). Soil conditions, such as pH, temperature, salinity, and drought level must be supportive for microbial growth (Zhang et al. 2020, Basu et al. 2021). Moreover, soil contamination due to pesticides and their residues, heavy metals, and other xenobiotics (Lutts and Lefèvre 2015) may have detrimental effects on the introduced beneficial agents. For example, the widespread utilisation of the herbicide glyphosate in various agricultural systems and practices raised the concerns of potential risk of glyphosate accumulation in different substrates (soils and underground water) with the consequent hazard for the environment and humans (Annett et al. 2014, Bai and Ogbourne

2016). As this herbicide has been reported to be toxic for various terrestrial and aquatic organisms, including beneficial soil microbes (Zobiole et al. 2011; Newman et al. 2016), the application of potential biofertilisers or biopesticides requires their ability to grow in the presence of this herbicide, presumably accumulated in agricultural soils (Singh and Walker 2006, Benbrook 2016).

Heavy metals and pesticides have significant impact on soil microbiota (Olaniran et al. 2013; Jaiswal et al. 2018). Natural adverse abiotic factors, including pH, salinity level, and water deficiency also have significant impact of the composition and abundance of the soil microbiome, as well as their activity (Shrivastava and Kumar 2015, Aslam et al. 2018, Wang et al. 2019).

Therefore, *in situ* strategies for the application of biofertilisers and biocontrol strains in agricultural soils containing residues of pesticides or heavy metals need to be well-considered, and the selection of beneficial microorganisms with enhanced tolerance to xenobiotics and adverse ecological factors is encouraged.

3.3.2. Abiotic stress factors caused by soil contamination

Soil contamination is a global problem. According to the Food and Agricultural Organization, soil contamination is «the addition of chemicals or materials to soil that have a significant adverse effect on any organism or on soil functions. A contaminant can be defined as any chemical or material out of place or present at higher-than-normal concentrations». Soils can become polluted in different ways: (1) depository for the storage of toxic substances; (2) mining; (3) remaining parts and residues of synthetic agricultural pesticides and fertilisers and even (4) radioactivity near thermal plants (FAO, Technical Summary, 2015). Among them, the wide use of synthetic chemicals in agriculture and industry correlate with the dispersal of them or their derivatives in the different environmental systems such as agricultural soil, underground water, aquatic environments, and air (Seech and Trevors 1991, Tani et al. 1998). Xenobiotics can pollute both soil and water, the fundamental strategic resources in agriculture (McGhee and Burns 1995).

According to the European Environmental Agency (2014), 340,000 different sites were contaminated in Europe with various xenobiotics, while more than 2.5 billion sites are estimated to be potentially polluted. In Australia, approximately 80,000 sites were recognized as polluted (Department of Environment and Conservation, Australia, 2010). In Asia, industrialization and urbanisation, as well as the intensive farming systems have also resulted in significant contamination. Wei and Chen (2001) reported that around 20 million hectares or 20% of all croplands are polluted by heavy metals in China.

Soil pollution by hazardous and toxic compounds is an extremely important issue. The adverse effects of xenobiotics, such as pesticides, heavy metals, and other pollutants remaining in soils can affect human health because of direct poisoning or indirectly, due to the accumulation of toxic elements in food chains (FAO, 2015). For instance, organic pollutants, such as polyaromaticaromatic hydrocarbons have toxic, mutagenic, and carcinogenic effect (Thavamani et al. 2012). Soil pollutants can inhibit the activity of microbial species important for plant growth and soil functions (Jaiswal et al. 2018, Vörös et al. 2019, Zhang et al. 2020). The utilisation of the globally-used herbicide glyphosate and further different pesticides, as well as the presence of heavy metals have significant influence on both indigenous soil microbiome and introduced beneficial microorganisms (Subsections 3.3.2.1, 3.3.2.2, and 3.3.2.3, respectively).

3.3.2.1 Soil pollution by glyphosate

Glyphosate (N-(phosphonomethyl) glycine) is a broad-spectrum herbicide globally used in crop production systems. It acts as a competitive inhibitor of 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS), which catalyses the synthesis of chorismic acid and its further conversion to prephenic acid and anthranilic acid, the precursors of aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan (Jaworski 1972, Steinrücken and Amrhein 1980, Boocock and Coggins 1983). Most bacteria use these aromatic amino acids to synthesize proteins, while plants are also able to involve these amino acids into the synthesis of phenylpropanoids, alkaloids, flavonoids, and other secondary metabolites (Weaver and Herrmann 1997). Glyphosate belongs to the group of phosphonates, distinguished by a strong carbon-phosphorous (C-P) bond, which makes the molecule chemically and thermally stable (Wanner and Metcalf 1992, Ermakova et al. 2017).

The agronomic efficiency, economic profit as well as the development and cultivation of glyphosate-resistant crops, such as soybeans, cotton, maize, canola, sugar beet, and alfalfa have resulted in the global agricultural utilisation of glyphosate-based herbicides (Baylis 2000; Gianessi 2005, Dill et al. 2008, Benbrook 2016) According to the data of Benbrook (2016), 747 million kg of glyphosate was used globally in agriculture in 2014, which is 14.6-times higher than the amount utilised in 1995. Additionally, 79 million kg were also used for non-agricultural purposes in 2014.

The extensive use of this herbicide raised concerns about its potential risks for ecosystems and human health as well (Borggaard and Gimsing 2008, Annett et al. 2014, Bai and Ogbourne 2016, Tarazona et al. 2017, Van Bruggen et al. 2018). Studies have declared the potential hazards of glyphosate for different organisms. Glyphosate exposure had detrimental effect on the populations or viability of various soil microorganisms, particularly *Acidobacteria* (Newman et al. 2016), Mn-reducing microorganisms, fluorescent pseduomonads, and indole acetic acid-producing rhizobacteria (Huber et al. 2005, Johal and Huber 2009, Zobiole et al. 2011), as well as rhizobial bacteria, arbuscular mycorrhizal fungi, dark septate endophytes, and free-living diazotrophs (Druille et al. 2015, 2016). Glyphosate was reported to decrease the reproductive rates of soil dwelling earthworms by 56% after its utilisation (Gaupp-Berghausen et al. 2015), and to reduce the number of lymphocytes and leukocytes resulting in weakened immune defence of silver catfish (*Rhamdia quelen*) (Kreutz et al. 2010, 2011). Glyphosate was discussed as a causal agent of microbiota shifting in many species, including mammals with potential consequences, such as neurological disorders (Van Bruggen et al. 2018, Rueda-Ruzafa et al. 2019). The toxic effect of glyphosate on *Enterococcus faecalis,* which neutralizes the neurotoxin of *Clostridium botulinum* in cow gut, was also revealed in *in vitro* studies (Krüger et al. 2013). Recently, Motta et al. (2018) found that the exposure of bees to glyphosate resulted in changes in their gut microbial communities - through the inhibition of the EPSPS enzyme of gut bacteria - and led to the reduced tolerance of bees to pathogens. Based on these findings, a similar effect of glyphosate on the human gut microbiome might be presumed. The LD_{50} of glyphosate (the amount causing the death of 50% of the tested population) for mammalian species is 3530-5600 mg/kg (Singh and Walker 2006). Humans from the non-agricultural sector might be exposed to glyphosate through the consumption of contaminated food (Bai and Ogbourne 2016). The extensive human exposure to glyphosate was confirmed by the detection of glyphosate residues in human urine samples (Krüger et al. 2014) and several studies have reported its negative effects on humans as well. Nuclear anomalies were detected in the buccal epithelial cells (Koller et al. 2012), and an increase in the incidence of chromosomal aberrations were found in human lymphocytes (Lioi et al. 1998) due to glyphosate. A case-control study has confirmed the association between glyphosate exposure and non-Hodgkin lymphoma (Eriksson et al. 2008). The International Agency for Research on Cancer included glyphosate

in group 2A – "probable carcinogenic to humans" - and declared it as "limited evidence in humans", stating "positive association observed between exposure to the agent and cancer, for which a causal interpretation is considered to be credible, but chance, bias or confounding could not be ruled out with reasonable confidence" (IARC, 2015, Tarazona et al. 2017).

Numerous studies have declared that the excessive use led to the spreading and accumulation of glyphosate and its residues in aquatic and soil ecosystems, resulting in the above mentioned risks for humans and the environment (Borggaard and Gimsing 2008, Annett et al. 2014, Bai and Ogbourne 2016, Van Bruggen et al. 2018). Glyphosate decomposition is regulated by both the activity and biomass of soil microorganisms (Von Wiren-Lehr et al. 1997, Araújo et al. 2003), in which various glyphosate-degrading bacteria and fungi are involved (Singh and Walker 2006, Fan et al. 2012, Kryuchkova et al. 2014, Manogaran et al. 2017, Zhan et al. 2018). However, the half-life of glyphosate in soil is estimated from 30 to 174 days (Singh and Walker 2006), which is a general vegetation time for most cultivated crops, when they can be amended with biological agents. Therefore, as glyphosate might persist in the soil during the entire cultivation period and inhibit the growth of the applied plant growth-promoting bacteria or biocontrol strains remarkably, the application of the potential biological agents requires the selection of strains with tolerance to glyphosate, its derivatives and residues.

3.3.2.2 The impact of pesticides on soil microbiome

The intensification of agriculture led to an increase in pesticide use to approximately two million tons annually including herbicides, insecticides, fungicides and others accounting for 47.5, 29.5, 17.5 and 5.5%, respectively (De et al. 2014, FAO, Main Report 2015). Pesticides and their residues can have significant effects on different types of soil microorganisms inhabiting agricultural fields (Arora and Sahni 2016). This effect highly depends on the microbial genera and species, as a pesticide might promote certain species while inhibiting other species at the same time. For instance, the application of mancozeb in the rhizosphere community of citrus resulted in an increase in the abundance of *Candidatus*, *Saccharibacteria*, *Parcubacteria*, and *Proteobacteria*, but the relative abundance of *Nitrospirae* was decreased (Huang et al. 2021). The utilisation of pesticides as nutrient source can also cause changes in the microbial population. During the biodegradation of aniline, the genera *Burkholderia* and *Pseudomonas*, and the authentic *Alcaligenes* became dominant, suggesting that they have the highest potential to utilise/decompose aniline (Tani et al. 1998). Pesticides have been reported to inhibit certain enzymes in terrestrial ecosystems. In particular, dehydrogenases and β-glucosidases, which mediate the microbial respiratory process and the transformation of soil organic matter, as well as phosphatases involved in the phosphorus cycle, were inhibited by various pesticides, such as captan, glyphosate, tebuconazole (dehydrogenase inhibition), endosulfan, glyphosate (β-glucosidase inhibition), and bromoxynil, acetamiprid, mancozeb, glyphosate, carbendazim (phosphates inhibition) (Riah et al. 2014).

These findings suggest that pesticides or their residues are abundant in various agricultural soils and might affect the microbial communities and their functions in either a positive or negative manner. Therefore, testing the beneficial strains for pesticide sensitivity is a crucial step before their *in vivo* utilisation.

3.3.2.3 The influence of heavy metals on bacteria

Various industrial and agricultural practices have led to the increased accumulation of toxic heavy metals in different areas (Lutts and Lefèvre 2015). Besides the well-known deleterious effects on ecosystems and human health (Jaiswal et al. 2018), heavy metals

represent an obstacle for the biodegradation of pollutants via the inhibition of the growth of bacteria (Chu 2018), including pesticide-degrading species (Olaniran et al. 2013, Zhang et al. 2020). The examination of the effect of Pb has revealed that the abundance of various beneficial soil microorganisms such N-fixing, ammonifying, P-solubilising and nitrosobacteria were increased in the presence of 500 but decreased by 1500 mg/kg Pb (Niu et al. 2019). According to the findings by Shi and Ma (2017), Cd had inhibitory effect on the respiratory intensity, as well as urease and catalase activity at a concentration-dependent manner. Cd and Pb also caused changes in the activity soil microbial population, in activities and the highest inhibitory effect was observed on bacteria compared to actinomycetes and fungi (Khan et al. 2010b). Five hundred mg/kg Ni caused 22.2% decrease in the relative abundance of various genera of methanogens (Xueping et al. 2020). All three tested heavy metals (Cd, Cr, and Pb) inhibited soil organisms, and the lowest concentrations that caused the adverse effect were 0.1-10 and 100 mg/kg for Cr as well as Cd and Pb, respectively (Martí et al. 2013).

Therefore, as polluted agricultural soils might contain heavy metals at concentrations that inhibit microbial growth, it is important to evaluate the metal tolerance of bacterial inoculants before their field application.

3.3.3 Natural abiotic stress factors in agricultural soils

Each agricultural soil is a complex, heterogeneous, and unique ecosystem combining the parental geological material, the presence and activity of the biota, as well as the history of its geomorphology and land use (Aislabie and Deslippe 2013). Currently, human activities, such as urbanisation, and climate change play tremendous role in soil changes (FAO, Main Report 2015). Climate change has resulted in an increasing risk of drought and heat stress, which are detrimental for certain crucial physiological processes of plants (Etesami and Maheshwari 2018, Fiodor et al. 2021). Soil salinity is another global concern affecting soil fertility and crop productivity. Saline soils contain excessive soluble salts (mainly NaCl and Na_2SO_4), causing reduced water content and elevated osmotic pressure, which harm crop growth. Soil salinization might occur due to both natural (rock weathering, accession of salts in marine sediments, atmospheric deposition) and human-derived (poor drainage system, the use of brackish groundwater in irrigation, etc.) factors (FAO, Main Report 2015). Drought and salinity stress, as well as pH level (acidic or alkaline soils) do not only limit plant development, but also have adverse influence on soil microorganisms (Bu et al. 2018, Li et al. 2021).

3.3.3.1 pH

Soils represent diverse environment, in which pH level should be favourable for the growth of plants and microorganisms. pH controls vital biogeochemical processes, such as trace element mobility, substance translocation, and the mobility of soil organic fractions, and also affects soil biological processes, including enzyme activities, the mineralization of organic matter, nitrification and denitrification (Neina 2019). Acidic and alkaline soils contain high and low amounts of H^+ in relation to OH^-, respectively. pH below 5.5-5.0 is acidic, while soils with high pH are defined as alkaline. Both acidic and alkaline pH resulted in adverse changes in the essential physico-chemical properties of soils, with detrimental consequences in terms of crop growth and productivity. As the solubility of nutrients also depends on soil pH, acidic and alkaline soils are unfavourable for farming, because low and high pH levels limit the uptake of elements (Neina 2019, Fiodor et al. 2021). Acidification does not only restrict soil fertility, but also increase the mobilization of Al and other heavy metals (FAO, Main Report 2015). Soil pH is also commonly recognized as an important

factor affecting the function and diversity of microbiota. Among 15 chemical soil properties, including the content of water, clay, loam, sand, and chemical elements, soil pH was found to be a dominant factor in determination of the structure of P-solubilising bacteria (Zheng et al. 2019). Čuhel et al. (2010) showed that the nature of denitrification end products was affected by pH level, in which the N_2O (N_2O+N_2) ratio was increased with decreasing pH due to the changes in the total denitrification activity.

Therefore, as certain microorganisms are viable in just a relatively narrow pH range, and soil pH has a significant impact on the diversity of soil-inhabiting bacteria (Wang et al. 2019), beneficial strains should be tested for their optimal pH range and applied in soils with corresponding pH values.

3.3.3.2 Salinity

Soil salinization is a worldwide issue occurring in more than 100 countries, the estimated total area of salt-affected soils was 412 million hectares (ha) in 2015 (FAO Main Report, 2015). The global area of soils impaired by salinity is growing by 10 million ha every year, which includes 1 million hectares within the European Union (Mokrani et al. 2020). Soil salinization often occurs in different regions of Central Asia, but it is also an issue in Spain, Turkey, Ukraine and Russia (FAO Main Report 2015). The area of salt-affected soils (including solonetz, alkaline soils and complexes of solonetz with other soils) in Hungary is estimated to be 0.56 (Tóth and Szendrei 2006), while in Kazakhstan approximately 111.5 million ha (Borovskii 1982). Several species of the *Bacillus*, *Pseudomonas*, *Rhizobium*, and *Enterobacter* genera have been reported to be potential strains to help to overcome salinity stress in plants (Shrivastava and Kumar 2015, Etesami and Maheswari 2018). Different mechanisms, such as the reduction of the stress hormone ethylene, production of IAA and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, the reduction of lipid peroxidation, and changes in ion uptake are involved in the alleviation of salinity stress by beneficial strains (Shrivastava and Kumar 2015). However, excessive salt concentrations also have adverse effect on bacterial growth. In the studies of Li et al. (2021), high salinity not only inhibited *Actinobacteria, Chloroflexi, Acidobacteria,* and *Planctomycetes,* but also suppressed certain crucial processes of N-metabolism (ammonification, N-fixation, nitrification, and denitrification). Microbial processes in soil were strongly inhibited by salinity, while the growth of bacteria and fungi was depressed in a concentration-dependent manner (Rath et al. 2016).

Therefore, as saline soils exist worldwide and cause significant crop reduction, testing the potential plant growth-promoting strains for the salinity tolerance is an important evaluation step necessary prior to their utilisation in fields.

3.3.3.3 Drought

Soil water is crucial for the functions of terrestrial ecosystems and well-being of humans. Despite only a small percentage of the world's water is concentrated in the soil, the fluxes of water through the soil are significant (FAO, Main Report 2015). Drought together with salinity represent the main abiotic stress factors, having adverse influence on physiological, biochemical, and molecular processes in plants with the consequence of crop reduction (Fiodor et al. 2021). Different *Bacillus*, *Pseudomonas*, *Enterobacter*, and *Flavobacterium* isolates have been reported in the literature to ameliorate drought stress in wheat, maize, cucumber, and rice. The common characteristics of these beneficial strains included IAA synthesis, the production of siderophores and hydrogen cyanide (HCN), as well as ACC deaminase activity (Etesami and Maheswari 2018). However, drought reduced the

labile organic C and N content of soil and caused a decreased relative abundance of Acidobacteria and Proteobacteria (Bu et al. 2018).

Hence, as drought stress represents a significant obstacle for bacterial growth and functions (Bu et al. 2018) and can also significantly inhibit plant growth and yield (Aslam et al. 2018, Devi et al. 2018), testing the drought tolerance of beneficial microorganisms is also an important step before their *in vivo* application.

4 OBJECTIVES

Taken the abovementioned facts into account, the primary objectives of the presented research have been defined as follows.

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

5 MATERIALS AND METHODS

5.1 Soil metagenome analysis

To assess the influence of the worldwide-used herbicide glyphosate on the microbial communities of soils, agricultural soil samples collected in Northern Serbia (Bácska/Bačka region) were subjected to metagenome analysis. The relevant characteristics of the samples are specified in Table 3.

Table 3. Characteristics of the examined soil samples

	Sample code	
	S5	**S9**
Location	Csantavér/Čantavir	Csantavér/Čantavir
Crop	tomato	tomato
Previous year's crop	potato	onion
Fertiliser	household compost	none
Pesticide	none	Glifol (glyphosate-containing herbicide)
Experimental application	***Control sample***	***Glyphosate-treated sample***

DNA extraction from the examined specimens, next-generation sequencing (NGS) and bioinformatical analysis was performed by the aid of an external service (SeqOmics Biotechnology Ltd., Hungary). The isolated total metagenome DNA was used for library preparation. *In vitro* fragment libraries were prepared using the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina. Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq® 500/550 High Output Kit v2 (300 cycles). Read numbers were 22740 and 8638 in the case of the control and the glyphosate-treated soil sample, respectively. Primary data analysis (base-calling) was carried out with Bbcl2fastq^ software (v2.17.1.14, Illumina). Reads were quality and length trimmed in CLC Genomics Workbench Tool 9.5.1 using an error probability of 0.05 (Q13) and a minimum length of 50 nucleotides as threshold. The composition of the bacterial communities detected in the control and glyphosate-treated soil was evaluated, and the glyphosate-treated sample (S9) was used for the subsequent isolation of potential glyphosate-tolerant bacteria.

5.2 Applied media, buffers, and culture conditions

Media (in the order of their appearance in the Materials and Methods section)

Solid Minimal Medium (Hatvani et al. 2013) – SMM (for 1 l):
1 g KH_2PO_4, 3 g Na_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 20 g agarose

Mineral Salt Medium (Ni et al. 2018) – MSM (for 1 l):
1 g NH_4NO_3, 0.5 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 1.5 g K_2HPO_4

Enrichment Medium (Vágvölgyi et al. 2013) – ER (for 1 l):
1 g glucose, 2 g Na_2HPO_4, 1 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 0.5 NaCl

Potato Dextrose Agar (VWR, Debrecen, Hungary) – PDA (for 1 l):
39 g PDA

Potato Dextrose Broth (VWR, Debrecen, Hungary) – PDB (for 1 l):
24 g PDB

Luria-Bertani (Bertani 1951) medium – LB (for 1 l):
7.5 g peptone, 2.5 g NaCl, 2.5 g yeast extract, 15 g agar

Standard Succinic Medium (Meyer and Abdallah 1978) – SSM (for 1 l):
6 g K_2HPO_4, 3 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g; 4 g $C_4H_6O_4$ (Reanal, Budapest, Hungary)

Pikovskaya Medium (Pikovskaya 1948) – PM (for 1 l):
10 g glucose, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.1 g/$MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.5 g yeast extract, 0.002 g $MnSO_4 \cdot H_2O$, 0.002 g $FeSO_4 \cdot 7H_2O$, 20 g agar, 5 g $Ca_3(PO4)_2$

Muromtsev's Medium (Kryuchkova et al. 2014) – MVM (for 1 l):
10 g glucose, 0.5 g $(NH_4)_2SO_4$, 0.2 g K_2SO_4, 0.4 g $MgSO_4$, 2.2 g $CaCl_2$, 3.8 gNa_2HPO_4, 0.5 g KNO_3, 0.1 g asparagine, 20 g agar, 5 g $Ca_3(PO4)_2$

Peptone Water (for 1 l) – PW:
10 g peptic digest of animal tissue (peptone meat, VWR, Debrecen, Hungary), 5 g NaCl

Supplemented Meat Medium (Kerekes et al. 2013) – MEE (for 1 l):
4 g beef extract, 4 g peptone, 10 g glucose, 1 g yeast extract

Chromobacterium violaceum Medium (Kerekes et al. 2013) – CVM (for 1 l):
1 g yeast extract, 10 g NaCl, 1 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.036 g Na-EDTA, 0.01 g ammonium iron (III) citrate, 10 g tryptone

Minimal Medium (Vágvölgyi et al. 1996) – MM (for 1 l):
1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 5 g $(NH_4)_2SO_4$

Yeast Extract Glucose (Vörös et al. 2019) medium – YEG (for 1 l):
2 g yeast extract, 2 g glucose

Buffers

50× TAE buffer (for 1 l):
242 g Tris base, 100 ml 0.5 M EDTA, 57.1 ml glacial acetic acid

1×TAE buffer (for 1 l):
20 ml 50×TAE buffer, 980 ml distilled water

Britton-Robinson buffer (Britton and Robinson 1931) (for 1 l):
Solution I: 0.04 M CH_3COOH, 0.04 M H_3PO_4, 0.04 M H_3BO_3
Solution II: 0.2 M NaOH

Phosphate buffer, pH 6.6 (for 1 l):
5.7 g KH_2PO_4, 4.4 Na_2HPO_4

Culture conditions

All incubations unless otherwise specified were performed at 25 °C.
All experiments were carried out in 3 replicates unless specified in the text.
Chemicals and reagents unless specified were purchased from Merck Ltd. (Budapest, Hungary)

5.3 Isolation and identification of glyphosate-tolerant bacteria

Glyphosate-tolerant bacterial strains were isolated from soil sample previously exposed to the glyphosate-containing herbicide "Glifol" (Galenika Fitofarmacija, Belgrade, Serbia), from the rhizosphere of tomato (*Solanum lycopersicum*) in Northern Serbia (Bačka region). Soil suspension was prepared by suspending 5 g soil in 50 ml sterile physiological saline solution (9 g/l NaCl in distilled water), and following serial dilution (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) prepared in saline solution, 50 µl of individual dilution step was spread on SMM (Hatvani et al. 2013) amended with 1 g/l glyphosate (*Glialka Star*, Monsanto Europe, Brussels, Belgium; active ingredient: glyphosate, 360 g/l) as the sole carbon and nitrogen source, and supplied with nystatin and carbendazim (0.1 g/l) to suppress fungal growth. Furthermore, two additional enrichment-culture techniques were applied. Fifty ml liquid MSM (Ni et al. 2018) and the same volume of liquid ER (Vágvölgyi et al. 2013) media prepared in 100-ml Erlenmeyer flasks, both supplemented with 0.1 or 1 g/l glyphosate as well as with 0.1 g/l nystatin and carbendazim, were used for inoculation with 0.5 ml soil suspension and incubated on a rotary shaker (100 revolutions per minute (rpm), MaxQ 8000 shaking incubator, Thermo Fisher Scientific, Marietta, OH, USA). After one-week incubation, shaken cultures of ER and MSM (1-ml) were inoculated to SMM as described above. Subsequently, 0.5 mL sample of MSM culture was inoculated in fresh MSM followed by one more week of incubation, and the subsequent inoculation to SMM. The inoculated SMM plates were incubated for 7-10 days. The colonies formed on SMM were considered to be glyphosate-tolerant bacteria, and single colonies were inoculated on PDA, which was further used for routine maintenance of bacterial strains. Overall, thirty bacterial strains were isolated and screened for their glyphosate-utilizing abilities. The isolates were stabbed on SMM amended with 1 g/l glyphosate, which was followed by a 7-day incubation. Based on the most intensive colony growth and different morphology, 10 isolates were selected for further studies and deposited in the Szeged Microbiology Collection (SZMC) (Table 10).

For species identification, cell suspensions (10^7 colony forming unit (CFU)/ml in 50 µl double distilled water (DDW)) prepared from overnight grown bacterial cultures were applied as DNA templates. The reaction mixture for polymerase chain reaction (PCR) contained 2 µl 10 × Taq Buffer with KCl and 20 mM $MgCl_2$, 2 µl 2 mM dNTP Mix, 0.1 µl 5 U/µl Taq DNA Polymerase (Fermentas), 4-4 µl 10 µM primers, 7 µl DDW, and 1 µl DNA template for each sample. The amplification of fragments of the 16S rRNA (Muyzer et al. 1993) and the beta subunit of RNA polymerase (*rpoB*) (Sajben et al. 2011) genes was performed in an MJ Mini Gradient Thermal Cycler (Bio-Rad, Foster City, CA, USA) under the conditions described in Table 4. The obtained PCR products were subjected to horizontal agarose gel electrophoresis using 1% agarose gel in 1 × TAE buffer (Section 5.2) for 15 min at 100 V and visualized by using UV-illumination. Automated sequencing of the amplicons was carried out using an external service (ELKH, Biological Research Centre, Szeged, Hungary). The obtained sequences were analysed by NCBI Basic local alignment search tool (BLAST) similarity search (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and alignments were made using the software ClustalX2 and GeneDoc.

Table 4. Primers and temperature profiles used for PCR

Primers (5'-3')	***Temperature profile***	***Reference***
16S rRNA gene		
Eub 341-F: CCTACGGGAGGCAGCAG Eub 1060-R: CGACACGAGCTGACGACA	95 °C, 2 min (1 cycle) 95 °C, 30 sec; 57 °C, 45 sec; 72 °C, 1 min (32 cycles) 72 °C, 7 min (1 cycle)	Muyzer et al. (1993)
RNA polymerase subunit beta (*rpoB*) gene		
rpob-PSF: AGTTCATGGACCAGAACAACC rpoB-PAR: CCTTCACGGTGAATTCGTTC	94 °C, 3 min (1 cycle) 94 °C, 1 min; 58 °C, 1 min; 72 °C, 1 min (30 cycles) 72 °C, 10 min (1 cycle)	Sajben et al. (2011)

Multiple sequence alignments of the 16S rRNA and *rpob* sequences were conducted using MAFFT v. 7.453 (Katoh and Standley, 2013) with the E-INS-i iterative refinement method. The alignments were concatenated and subjected for phylogenetic inference. Phylogenetic reconstruction was carried out by using IQ-TREE v. 1.6.12 (Nguyen et al. 2015) with the K2P+G4 model for the 16S and HKY+F+G4 model for the rpob dataset determined by the inbuilt ModelFinder tool (Kalyaanamoorthy et al. 2017). Statistical support of the best tree was calculated with Ultrafast bootstrap approximation (Hoang et al. 2018) with 5000 replicates.

5.4 Examination of the plant growth-promoting potential of glyphosate-tolerant bacteria

5.4.1 Enhancement of tomato growth

The potential plant growth-promoting effect of the isolated glyphosate-tolerant bacterial strains was assayed using tomato (*S. lycopersicum*) seeds (cultivar «Bohun», Garafarm, Hungary). Seed surface was sterilised by soaking the seeds in 70% ethanol shaken for 1 min, followed by washing 3 times with sterile distilled water. Thereafter, the seeds were treated in 1.5% NaOCl for 10 min and rinsed with sterile distilled water 5 times. Fifty seeds were soaked in 30 ml 48-hour bacterial cultures grown in PDB as well as in controls (non-inoculated sterile PDB and sterile distilled water). After two-hour soaking, 10 seeds were randomly selected and transferred on sterile paper disks saturated with 5 ml sterile distilled water in Petri dishes, placed in a versatile environmental test chamber (MLR- 352H, Panasonic Healthcare, Sakata, Japan) and incubated at average humidity of 60-70% and temperatures of 18-20 °C and 25-27 °C during night and daytime, respectively (Xu et al. 2014). To mimic natural soil conditions, no artificial light was applied. Seed germination, fresh mass, and seedling length were measured on the 7th day of incubation. The germination rate of seeds was calculated as the ratio of the number of germinated seeds to the total number of the tested seeds, multiplied by 100, then, seedling and root lengths of each plant were measured. Non-germinated seeds were included as 0 values for each measurement. Vigor index was calculated based on the total length and germination rate using the formula suggested by Anupama et al. (2014):

Vigor index = (Length of seedling, cm + Length of roots, cm) × germination rate, %

5.4.2 Indole-3-acetic-acid biosynthesis

The potential ability of the bacterial isolates to produce IAA was initially screened on solid agar plates using the method reported by Shrivastava and Kumar (2011), with minor modifications. Sterile cork-borer was used to make holes (1 cm in diameter) in PDA plates supplied with 1 g/l L-tryptophan as IAA precursor. Two hundred µl of overnight bacterial cultures grown in 30 ml PDB were pipetted into the holes. Empty holes and holes filled with sterile PDB served as the negative controls. After 48-hour incubation, the growing bacterial cultures were removed from the surface of the medium with tissue paper, and 200 µl Salkowski reagent containing 12 g/l $FeCl_3$ dissolved in 37% H_2SO_4 (Bric et al. 1991) was pipetted into each hole. The development of pink colour around the holes was the indicator of the reaction of IAA produced by bacteria with Salkowski reagent.

For the quantitative confirmation of the IAA-synthesising ability of the isolates, the colourimetric assay introduced by Gutierrez et al. (2009) was applied with modifications. A loopful of overnight culture grown on PDA was inoculated separately in 5 ml PDB and the same volume of PDB supplied with 1 g/l L-tryptophan. Non-inoculated PDB with or without L-tryptophan served as negative control. After 72-hour of incubation, 2 ml samples were taken in Eppendorf tubes (2-ml) and centrifuged at 6,000 *g* for 10 min. Thereafter, 1 ml supernatant was mixed with 1 ml Salkowski reagent, and the mixture was incubated in the dark for 20 min. The appearance of a pink colour indicated the presence of IAA in the culture supernatant, and the optical density (OD) was measured at 530 nm (OD_{530}).

High performance liquid chromatograph (HPLC) coupled to a mass spectrometer (MS) was applied to determine the concentration of IAA produced in the culture supernatants according to Khan et al. (2016) with modifications. The strains were inoculated into 50 ml PDB supplied with L-tryptophan (1 g/l) and the same volume of PDB without tryptophan at the initial concentration of 10^5 CFU/ml. Seventy-two-hour shaken cultures (25 °C, 130 rpm, MaxQ 8000 shaking incubator, Thermo Fisher Scientific, Marietta, OH, USA) were transferred into 50-ml Falcon tubes, and centrifuged (Heraeus Biofuge Primo centrifuge, Thermo Fisher Scientific, Osterode, Germany) at 6,000 *g* for 15 min. Obtained 40 ml cell-free culture supernatants were divided into two 50-ml Falcon tubes (20 ml in each) and 1 M HCl was used to adjust pH to 2-3. Extraction was performed 3 times using 25, 10, and 10 ml ethyl acetate, respectively. Each time after the addition of ethyl acetate to the supernatant, the mixture was shaken thoroughly for approximately 1 min and subjected to centrifugation at 6,000 *g* for 5 min. The extracts from the first, second, and third extraction steps (20, 10, and 10 ml, respectively) were combined and evaporated at 25 rpm, 40 °C using an IKA HB10 basic rotary evaporator (IKA, Staufen, Germany). The residues were re-dissolved in 1 ml methanol (MeOH) of HPLC super gradient grade and stored at −20 °C. On the day before the HPLC measurements, the samples were stirred up by vortexing, and centrifuged (Heraeus Fresco 17 centrifuge, Thermo Fisher Scientific, Osterode, Germany) at 9,600 *g* for 10 min. After centrifugation, 500–700 µl of each sample was transferred into HPLC vials and kept overnight at 4 °C prior to analysis. HPLC-MS measurements were carried out using a Shimadzu HPLC system, containing a DGU-20A5R degasser, an LC-20ADXR pump and an SIL-20AXR autosampler coupled with Thermo Scientific TSQ Quantum Access MS (Thermo Fisher Scientific, Waltham, MA, USA). Eluent A was H_2O + 0.1% formic acid, while acetonitrile + 0.1% formic acid served as eluent B. During the measurements, gradient elution was applied with the following gradient program: min/B%: 1/20, 3.04/37, 3.2/95, 7.2/95, 7.4/20, 11.4/20. The flow rate was 0.3 ml/min, and a Phenomenex Gemini-NX C18, 50 × 2 mm, 3 µm chromatographic column was used. The injection volume was 5 µl in each case, and the column oven temperature was 40 °C. For the MS detection of IAA, electrospray ionization (ESI) was used in positive mode. The spray voltage, the vaporizer temperature, the sheath gas pressure, the aux gas pressure, and the ion transfer capillary temperature were

4000 V, 380 °C, 20 arbitrary unit, 55 arbitrary unit, and 250 °C, respectively. The measurements were performed in selected reaction monitoring mode (SRM) with the following transitions: 176- > 130 a mass-to-charge (*m/z*) ratio (quantifier), 176- > 103 *m/z* (qualifier). Calibration standard solutions were prepared in MeOH. An eight-point calibration curve was fit in the range of 0.01–5.0 μg/ml. The lower limit of quantification (LLOQ) was 0.01 μg/ml, while the upper limit of quantification (ULOQ) was 5.0 μg/ml. Samples with higher IAA concentration than the ULOQ were tenfold diluted.

5.4.3 Siderophore production

Siderophore assay was carried out using the blue agar Chromeazurol S (CAS) dye technique (Schwyn and Neilands 1987), which was modified according to Lakshmanan et al. (2015). CAS formulation consists of the mixture of 3 solutions, which was prepared as follows: Ten ml $FeCl_3$ solution (1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl) was added to 50 ml CAS solution (2 mM CAS in distilled water), then 40 ml HDTMA solution (5 mM hexadecyltrimethylammonium bromide in distilled water) was also added to the mixture. Fifty ml sterile CAS formulation was mixed with 450 ml sterile LB medium. The prepared CAS agar plates were inoculated with bacterial strains pre-grown on PDA overnight, and incubated for 7 days in darkness at 30 °C. The development of brown-orange halo zone around the bacterial colonies was the sign of siderophore production.

For the quantitative determination of the produced siderophore percentage, the spectrophotometric analysis described by Arora and Verma (2017) was applied. All glassware was soaked overnight in 10% HNO_3 (Santos et al. 2014), then rinsed with distilled water twice before use in order to prevent iron contamination. The strains were grown in iron-free SSM (Meyer and Abdallah 1978). SSM amended with $FeCl_3 \cdot 6H_2O$ (0.029 g/l) was used as positive control (iron-replete samples), while non-inoculated SSM served as reference sample. Prior to sterilization, pH was adjusted to 7.0 using 0.2 M NaOH. Bacterial suspensions were prepared in 1 ml physiological saline solution at 10^8 CFU/ml from cultures grown on solid iron-free SSM (amended with agarose, 20 g/l) for 48 hours. Eppendorf tubes (1.5-ml) containing 0.5 ml liquid SSM with or without $FeCl_3 \cdot 6H_2O$ were inoculated with 5 μl bacterial suspensions, except for reference samples, and they were incubated at 25 °C for 48 hours. After incubation, the tubes were centrifuged at 9,600 *g* for 10 min, and 100 μl cell-free supernatant was transferred into separate wells of a 96-microtiter plate. Equal amount of CAS solution was added in the wells and the mixtures were homogenised using a pipette. The CAS solution appears as strong blue-coloured compound, which becomes colourless if the siderophores present in culture supernatant remove Fe from the solution. The mixtures were incubated in darkness for 20 min, and analysed with a SPECTROstar Nano microplate reader (BMG Labtech, Offenburg, Germany) at OD_{630}. The obtained OD_{630} values were used to calculate the percentage of siderophore production according to the following formula:

$$\frac{Ar - As}{Ar} \times 100\ \%$$

where *Ar* is the absorbance of the reference sample, and *As* is the absorbance of the inoculated sample (Payne 1994).

5.4.4 Phosphorus solubilisation

The potential phosphorus-solubilising ability of the bacteria was examined on PM (Pikovskaya 1948) and MVM (Kryuchkova et al. 2014) media, both amended with 5 g/l $Ca_3(PO_4)_2$ as insoluble phosphate source. The bacterial strains were stabbed on the agar

plates, 3 strains per plate, and the halo zone formation around the appearing colonies, indicating phosphate solubilisation, was monitored during the two-week incubation.

5.4.5 Ammonia production

The assay was carried out according to Goswami et al. (2015) using the Nesslerization method. For the preparation of Nessler's reagent, 50 g KI was dissolved in 50 ml cold distilled water. Subsequently, the saturated solution of $HgCl_2$ (22 g in 350 ml distilled water) was added until the formation of a precipitate. Two-hundred ml 5 N NaOH was added to the solution and made up to the final volume of 1 l by the addition of distilled water. A loopful of bacterial culture pre-grown overnight on PDA was inoculated in 15-ml test tubes containing 5 ml PW. Non-inoculated PW served as negative control. After incubation for 7 days at 25±2 °C, 2 ml culture was transferred into Eppendorf tubes (2-ml) at the stationary phase of the bacterial growth and centrifuged at 10,000 *g* for 10 min. One ml supernatant was mixed with 1 ml Nessler's reagent. The change of colour from brown (natural for peptone water) to yellow was considered as the reaction between Nessler's reagent and ammonia produced by the bacteria. The mixture was diluted up to 10 ml with 8 ml distilled water, and 200 µl was used for spectrophotometric analysis at OD_{450}.

A dilution series of $(NH_4)_2SO_4$ (10, 9, 8, 7, 6, 5, 4, 3, 2, and 1 µmol/ml) was prepared in PW for framing up the calibration curve based on the reaction of $(NH_4)_2SO_4$ with Nessler's reagent. $(NH_4)_2SO_4$-free PW mixed with Nessler's reagent was used as negative sample.

5.4.6 Statistical analysis

Assumptions for parametric tests as normal distribution of the residuals and homoscedasticity (similar variances in all groups) were checked before applying statistical test. The effect of bacteria on tomato growth parameters (seedling length, root length, seedling+root length, fresh mass, vigor index and germination rate) was analysed by one-way Analysis of Variance (ANOVA). Each significant ANOVA result ($p<0.05$ level) was subjected further to post-hoc pair-wise comparisons (Tukey's HSD (honestly significant difference) test). The variants not corresponding to the mentioned assumptions for parametric tests were analysed using non-parametric alternative for ANOVA Kruskal-Wallis test. ANOVA or Kruskal-Wallis test was also applied to verify the significance of OD_{530} values for IAA colourimetric test to select strains with high OD_{530} values for the HPLC assays. All statistical analyses and graphical visualization of the data presented in this section were performed using R (version 3.5.1; https://www.r- project.org/) and RStudio Desktop software (http://www.rstudio.com/), with *ggplot2* (Wickham 2016) and *patchwork* (Pedersen 2020) packages; multiple comparisons of treatments (Tukey's HSD test) were performed with *agricolae* package (Mendiburu 2020). Unless specified, values throughout the text are presented as means of three replicates ± standard deviations (SD). Image processing, including cropping, scaling, and editing was also performed in R with *magick* package (Ooms 2021).

5.5 Investigation into the biocontrol potential of glyphosate-tolerant bacteria

5.5.1 Anti-quorum sensing activity

Chromobacterium violaceum SZMC 6269 and *Serratia marcescens* SZMC 0567 retrieved from the SZMC collection were studied as model organisms for anti-quorum sensing activity test using agar diffusion method (Kerekes et al. 2013). Glyphosate-tolerant bacterial strains were incubated in 50 ml PDB for 3 days at 25 °C, 130 rpm (MaxQ 8000

shaking incubator, Thermo Fisher Scientific, Marietta, OH, USA). The cultures were transferred into 50-ml Falcon tubes and centrifuged at 3,000 *g* for 10 min (Heraeus Fresco 17 centrifuge, Thermo Fisher Scientific, Osterode, Germany). The obtained supernatants were at −20 ºC and thawed before using. Bacterial suspensios were prepared in physiological saline solution from 24-hour cultures pre-grown at 30 °C on CVM and MEE media in the case of *C. violaceum* and *S. marcescens*, respectively. The media were sterilised and cooled down to 40 °C, then the suspensions of the *C. violaceum* and *S. marcescens* strains were added to corresponding ones at 10^6 CFU/ml concentration, thoroughly mixed, and poured in Petri dishes (9 cm in dameter). The plates were left for surface air drying for 10 min under sterile conditions, then holes (0.8-1.0 cm diameter) were cut in the solidified media using a sterile cork-borer, and filled with 100, 200, and 300 µl supernatant of glyphosate-tolerant strains. The plates were incubated for 5 days at 30 °C, the appearance of halo zones was the sign of anti-quorum sensing activity.

5.5.2 *In vitro* antagonistic activity towards plant pathogenic fungi and bacteria

Dual-culture method was applied for the determination of the potential antagonistic activity of the glyphosate-tolerant bacteria against different fungal and bacterial plant pathogens (Table 5).

Table 5. List of plant pathogenic fungal and bacterial strains used in the studies of the biocontrol potential of glyphosate-tolerant bacteria

Species	**SZMC number**
Fungi	
Alternaria solani	62417
Botrytis cinerea	21047
Colletotrichum gloeosporioides	16086
Fusarium culmorum	11039
Fusarium graminearum	11030
Fusarium solani	16084
Gaeumannomyces graminis	23658
Phoma cucurbitacearum	16088
Sclerotinia sclerotiorum	62507
Bacteria	
Agrobacterium tumefaciens	21407
Corynebacterium michiganense	0016
Erwinia carotovora	6189
Pseudomonas syringae	16160
Xanthomonas campestris	6182

Agar plugs (5 mm in diameter) cut from the actively growing edge of the fungal colonies were placed at the centre of PDA plates. Overnight cultures of the glyphosate-tolerant isolates pre-grown on PDA were stabbed with sterile toothpicks at 1-1.5 cm distance away from the inoculated fungi, three strains per plate. Plates inoculated with solely the fungal or bacterial strains served as controls. The plates were monitored daily during a 7-day incubation period. Attenuation of mycelial radial growth in the direction of the bacterial colony was the result of the antagonistic effect of the bacteria towards the fungal pathogens.

For testing the potential antagonistic effect of glyphosate-tolerant strains against bacterial plant pathogens (Table 5), suspensions prepared from overnight cultures of the target pathogens in sterile physiological saline solution were added at 10^6 CFU/ml

concentration to sterilised PDB medium amended with 1% agar after cooling to 40 °C. The thoroughly mixed medium was poured in Petri dishes and left open for air-drying for 10 min under sterile condition. Suspensions of the glyphosate-tolerant strains were prepared the same way as described for plant pathogens at 10^7 CFU/ml, and 10 µl was inoculated on the surface of the solidified medium (three strains per plate). The plates were air-dried for additional 10 min and incubated for 7 days. Positive controls included plates inoculated with pathogenic culture alone and plates inoculated with glyphosate-tolerant strains only. The appearance of halo zones around the inoculation spots of the glyphosate-tolerant isolates were regarded as the inhibitory potential of the glyphosate-tolerant isolates. Pathogenic strains found to be negatively affected by the glyphosate-tolerant strains were selected for further biocontrol assays described below.

The subsequent biocontrol assays were performed with 7 *A. tumefaciens* (SZMC 14554, SZMC 14555, SZMC 14556, SZMC 14557, SZMC 21395, SZMC 21407, and SZMC 21783) and 7 *A. vitis* (SZMC 21396, SZMC 21397, SZMC 21398, SZMC 21784, SZMC 21785, SZMC 21786, and SZMC 21787) strains. All *Agrobacterium* strains were grown on PDA (Wise et al. 2006). Fresh cultures pre-grown on PDA for 24 hours were used to prepare bacterial suspensions in physiological saline solution at 10^6 and 10^7 CFU/ml in the case of the *Agrobacterium* and glyphosate-tolerant isolates, respectively. PDA plates were flooded with 5 ml of the *Agrobacterium* suspensions of strains to cover the full surface. The remaining suspension was removed, the plates were air-dried for 20 min, then 10 µl suspension of the glyphosate-tolerant isolates was inoculated on the surface (three strains per plate), then the plates were air-dried for further 10 min. Plates inoculated solely with the *Agrobacterium,* or the glyphosate-tolerant isolates served as controls. The inoculated plates were incubated for 3 days and monitored for the appearance of halo zones around glyphosate-tolerant strains, which was associated with activity. Glyphosate-tolerant isolates with the highest inhibition potential and the most vulnerable *Agrobacterium* strains were selected for the subsequent biocontrol studies (Subsections 5.5.3-5.5.9).

5.5.3 Carbon source utilisation tests

A. tumefaciens SZMC 14557, *A. vitis* SZMC 21396 as well as the glyphosate-tolerant bacterial isolates *E. adhaerens* SZMC 25856, *P. resinovorans* SZMC 25872, and *P. resinovorans* SZMC 25875 were tested for their ability to utilise various compounds as the sole carbon source on MM (Vágvölgyi et al. 1996) amended individually with 80 different substances (Table 6).

Table 6. List of compounds tested as the sole carbon sources

№	Carbon source
1	(-) quinic acid
2	2-keto-D-gluconic acid
3	adenosine
4	alpha-methyl-D-mannoside
5	ascorbic acid
6	beta-alanine
7	beta-methyl-D-galactoside (1-O-methyl-β-galactopyranoside)
8	cellobiose
9	cis-aconitic acid
10	cytidine
11	cytosine
12	D-arabinose
13	dextran

14	D-fructose
15	D-galactose
16	D-glucuronic acid
17	D-glucosamine
18	D-glucose
19	dihydroxyacetone
20	DL-isocitric acid
21	D-lyxose
22	D-mannitol
23	D-mannose
24	D-xylose
25	ethanol
26	fumaric acid
27	galactitol
28	gallic acid
29	gamma-butyrolactone
30	gentisic acid
31	gluconic acid
32	glycerol
33	glycerol-l-monoacetate
34	glycine
35	i-erythritol
36	inosine
37	inulin
38	ketoisovaleric acid
39	lactose
40	L-alanine
41	L-arabinose
42	L-arginine
43	L-asparagine
44	L-citrulline
45	L-glutamic acid
46	L-glutamine
47	L-histidine
48	L-isoleucine
49	L-lactic acid (Na-DL-lactate)
50	L-lysine
51	L-malic acid
52	L-methionine
53	L-ornithine
54	L-proline
55	L-rhamnose
56	L-serine
57	L-sorbose
58	L-threonine
59	L-tryptophan
60	L-valine
61	maltose
62	melezitose

63	melibiose
64	myo-inositol
65	nicotinic acid
66	p-arbutin
67	protocatechuic acid (3,4-dixydroxybenzoic acid)
68	pyruvic acid (sodium pyruvate)
69	raffinose (raffinose pentahydrate)
70	ribitol (adonitol)
71	sorbitol
72	starch
73	succinic acid
74	sucrose
75	tannic acid
76	thymine
77	uridine
78	vanillin
79	xylan
80	xylitol

The concentration of each carbon source in MM was 2 g/l, and the stock solutions of the tested carbon sources were prepared in double concentrated form (4 g/l) in sterile distilled water. For liquid carbon sources (ethanol, gamma-butyrolactone, glycerol, and glycerol-l-monoacetate), 6 µl of the corresponding compound was dissolved in 1.5 ml sterile distilled water to obtain the desired concentration. The sterility of the prepared stock solutions was checked by inoculating 5 µl amounts on PDA followed by incubation for 3 days.

The test was carried out using broth microdilution technique. One-hundred µl sterile MM was added in wells of a 96-well microtiter plate, and mixed with 100 µl of the stock solutions to obtain the final concentration 2 g/l. Bacterial suspensions were prepared in 20 ml physiological saline solution at 10^6 CFU/ml, and 20 µl was inoculated in each well to obtain the initial cell concentration 10^5 CFU/ml. Non-inoculated negative samples amended with 20 µl sterile physiological solution were used to examine any possible increase in OD_{620} due to the tested compounds. The microtiter plates were incubated for 48 hours, then the cell density was determined (OD_{620}).

5.5.4 The influence of carbon sources on the antagonistic potential of glyphosate-tolerant bacteria against *A. tumefaciens*

To study the influence of different carbon sources on the biocontrol activity of glyphosate-tolerant bacteria against *A. tumefaciens*, MM medium (Vágvölgyi et al. 1996) was amended with 13 compounds individually (2 g/l) as the sole carbon source. Based on the results of previous studies (Subsection 5.5.3), the applied substances were 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, which were utilised by *P. resinovorans* (genotype I) SZMC 25872 and *P. resinovorans* (genotype II) SZMC 25875 as the most promising biocontrol isolates, and *A. tumefaciens* SZMC 14557 as the most susceptible strain (Subsection 5.5.2). The assays were carried out as described in Subsection 5.5.2. As the initial assays for testing the biocontrol potential of the isolates was performed on PDA (Subsection 5.5.2), this medium served as the positive control to compare the effect of carbon sources on the growth inhibition of *A. tumefaciens*.

5.5.5 Testing the inhibitory effect of the cell-free culture filtrates of *P. resinovorans* on the growth of *A. tumefaciens*

The glyphosate-tolerant bacteria were cultivated in PDB, LB, YEG (Vörös et al. 2019), and MM (Vágvölgyi et al. 1996) supplied individually with glucose, L-alanine, succinic acid, and sodium pyruvate as sole carbon source, which resulted in the highest biocontrol activity in the previous experiments (Subsection 5.5.4). Prior to sterilization, the pH of all media was adjusted to 6.8-6.9 using 1 M NaOH, except for PDB, which was used without pH adjustment, and YEG, with its initial pH 6.8. Suspensions of the glyphosate-tolerant *E. adhaerens* SZMC 25856, *P. resinovorans* SZMC 25872, and *P. resinovorans* SZMC 25875 strains (overnight-grown on PDA) were prepared in 5 ml sterile physiological saline solution, which were used to inoculate 50 ml of the different media in 100-ml Erlenmeyer flasks at 10^5 CFU/ml starting concentration. Non-inoculated flasks containing the same amount of the corresponding media served as the negative controls. The cultures were incubated on a rotary shaking incubator (MaxQ 8000 shaking incubator, Thermo Fisher Scientific, Marietta, OH, USA) at 130 rpm, 28 °C for 3 days, and the cell density (OD_{620}) was determined by using a spectrophotometer (SPECTROstar Nano microplate reader, BMG Labtech, Offenburg, Germany) on the final day of incubation. The obtained cultures were transferred to 50-ml sterile Falcon tubes and centrifuged at 8,500 rpm for 10 min (Heraeus Biofuge Primo centrifuge, Thermo Fisher Scientific, Osterode, Germany). Thereafter, approximately 5-7 ml of the obtained supernatants were filtered through 0.22-µm filter membrane (Millex GV, syringe-driven filter unit, Merck, Tullagreen, Carrigtwohill, Cork, Ireland) to remove all remaining bacterial cells. The sterility of the obtained cell-free culture filtrates (CCF) was checked by inoculating 5 µl on PDA, followed by 3 days incubation of and daily observation for any bacterial growth. The obtained CFF samples and the remaining supernatants were stored at −20 ºC and thawed before use.

Broth microdilution method, using a 96-well microtiter plate was applied to study the inhibitory effect of the obtained CCF samples on the growth of *A. tumefaciens* SZMC 14557. PDB medium was amended with the different CCFs at 50, 25, and 12.5%, concentrations, in the total volume of 100 µl. Overnight culture of *A. tumefaciens* grown on PDA was used to prepare suspension in sterile PDB medium at 2×10^6 CFU/ml concentration, and 100 µl was added to the wells of the microtiter plate already containing the diluted CCFs to obtain the 10^6 CFU/ml starter cell concentration. Inoculated PDB without CCF and non-inoculated PDB amended with CCFs in the mentioned concentrations served as the positive and negative controls, respectively. The plates were incubated for 24 hours, and the OD was measured at OD_{620} using a SPECTROstar Nano microplate reader (BMG Labtech, Offenburg, Germany). The growth inhibition rate caused by the CCFs was determined as the difference between the positive control and treated samples multiplied by 100% and divided with the value of positive control. The obtained values of the growth inhibition were subjected to correlation analysis with the data of siderophore amount (OD_{630}, Subsection 5.5.6). CCF samples with the significant inhibition rate ($p<0.05$) were used for investigating possible modes of action involved the growth inhibition of *A. tumefaciens* (Subsection 5.5.7).

5.5.6 Siderophore production by *P. resinovorans* strains cultivated in different media correlated with the growth inhibition of *A. tumefaciens*

Determination of siderophores in the obtained CCF samples was carried out based on the colourimetric reaction between CAS solution and CCF as described in Subsection 5.4.3. Correlation analysis with the inhibitory effect was performed according to Subsection 5.5.10.

5.5.7 Testing the hypothesis about the siderophore- and ROS-mediated suppression of the growth of *A. tumefaciens* and the inhibitory effect of CCF after heat-treatment

CCF samples obtained from MM amended with L-alanine and succinic acid at the concentrations that caused the most remarkable growth inhibition (25 and 50%) were tested to evaluate the possibility of siderophore or ROS-induced suppression of *A. tumefaciens* SZMC 14557 using the method described earlier (Subsection 5.5.5). Ten µl $FeCl_3 \cdot 6H_2O$ from 0.58 mg/ml stock (final iron concentration in sample 29 mg/l, Santos et al. 2014) or 10 µl ascorbic acid from 3.4 mg/ml (final concentration in sample 1 mM/0.17 mg/ml, Adler et al. 2012) were both dissolved in distilled water and added into treated samples to eliminate possible iron sequestration or ROS-generation, respectively, because of the presence of CCF. Samples without addition of an extra iron source or ascorbic acid were amended with 10 µl sterile distilled water. The inhibitory effect of CCF after heat treatment was also tested with the same method. To eluminate the tentative inhibitiory effect of metabolites detected in succinic acid, CCF were heated at 80 °C and 90 °C for 60 and 30 min, respectively (Sakr et al. 2013). Non-heated samles were served as positive control to compare the inhibition rate with heated samples.

5.5.8 Extracellular enzyme activity assays

Extracellular enzyme activities in the CCF samples of the glyphosate-tolerant strains grown in different media (Subsection 5.5.5) were determined using chromogenic substrates (Table 7), following the protocol described by Vörös et al. (2019).

Table 7. Chromogenic substrates used to test extracellular enzyme activities

Extracellular enzyme system	**Substrate**
Chymotrypsin-like proteases	*N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide
Trypsin-like proteases	*N*-Bz-L-Phe-L-Val-L-Arg-p-nitroanilide-hydrochloride
Esterases	p-nitrophenyl-palmitate

The substrates were dissolved in dimethyl sulfoxide to obtain 3 mM concentration, while phosphate buffer was prepared in distilled water. Fifty µl of the CCF samples and 50 µl phosphate buffer (Section 5.2) were added in the wells of a 96-well microtiter plate and mixed with 50 µl the corresponding chromogenic substrate (final concentration 1 mM); then the mixture was incubated for 20 min. The OD of the reaction mixtures was measured at 405 nm (OD_{405}).

5.5.9 MS-MS analysis and identification of the bioactive metabolites potentially involved in the biocontrol of *A. tumefaciens*

To detect and identify the main bioactive compounds, culture supernatants of the glyphosate tolerant bacteria suppressing the growth of *A. tumefaciens* (Subsections 5.5.5 and 5.5.7) were subjected to High-performance liquid chromatograph-high-resolution mass spectrometry (HPLC-HRMS) analysis. Bacterial broth cultures obtained as described in Subsection 5.5.5 were analysed for the identification of potential active metabolites which had the inhibitory effect on the growth of *A. tumefaciens* SZMC 14557 (Subsections 5.5.5 and 5.5.7). Liquid cultures (2.6 ml of each sample collected in two 1.5-ml Eppendorf tube, 1.3 ml in each tube) were centrifuged for 10,000 *g* for 10 min and 0.9 ml supernatant from each tube was combined in a new 2-ml Eppendorf tube. Centrifugation at 10,000 g for 10 min

was repeated on the obtained supernatant and 0.5 ml was taken and subjected for overnight freeze-drying. Twohundred µl of 80% MeOH was added in the lyophilized residues followed by soaking in an ultrasound bath (80 Hz, 25 °C, Elmasonic P, Singen, Germany) for 10 min. Then, samples were stirred up thoroughly with a vortex for 15 sec and subjected in ultrasonic bath for 5 min more. After that, samples were homogenised with a brief vortexing and centrifuged (5430R, Eppendorf, Hamburg, Germany) at 18,000 *g* and 20 °C for 10 min. Sixty µl of the supernatant was transferred in the HPLC vials and prior to analysis, each sample was amended with 5 µl of the internal standard at 0.1 mg/ml concentration (buspirone, stock at 1 mg/ml dissolved in MilliQ water). This was followed by brief vortexing and the samples were subjected to HPLC-HRMS analysis.

All samples were analysed in both positive and negative ionization modes of HPLC-HRMS. The measurements were performed using a DionexUltimate 3000 UHPLC system (Dionex) coupled to an Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer operating with a heated electrospray interface (HESI). The metabolites were separated using a Gemini NX C18 (3 µm, 150 x 2 mm) column. For separation of metabolites, water (A) and MeOH/acenonitrile (1/1) both supplemented with 0.1% formic acid served as mobile phases. The linear gradient was executed as follows: 5% solvent B 0–2 min; increased to 95% B 2–13 min; 95% B until 19 min; 5% B 19–19.5 min and 5% B until 24 min. The flow rate was 0.2 ml/min, the injection volume was 3 µl and the column temperature was maintained at 25 °C. The capillary and the heater temperatures were 320 and 300 °C, respectively. The sheath gas and the auxiliary gas flow rates were applied at 30 and 10 (in arbitrary units), respectively. The precursor ion scan was done in full MS mode at a resolution of 70,000 at *m/z* value of 200 (3 scans/s), auto gain control (AGC) target of 3e6, maximum injection time (IT) of 100 ms, and a scan range of *m/z* values of 100-1500. The product ion scan was conducted in a data dependent MS^2 mode ($ddMS^2$) using a resolution of 17,500 at a *m/z* value of 200, AGC target of 2e5, maximum IT of 200 ms, normalized collision enegry (NCE) of 30 eV, stepped NCE of 50%. Isolation window was 0,4 *m/z*. HPLC-HRMS data were acquired using Trace Finder 4.0 software. The raw MS data files were processed using Compound Discoverer™ (2.1) software.

5.5.10 Statistical analysis

Assumptions for parametric tests as normal distribution of the residuals and homoscedasticity (similar variances in all groups) were checked before applying statistical test. The effect of the CCF samples on cell density of *A. tumefaciens* SZMC 14557 (OD_{620}) was analysed by one-way ANOVA, followed by post-hoc pair-wise comparisons (Tukey's test). The variants not corresponding to the mentioned assumptions for parametric tests were analysed using non-parametric alternative for ANOVA - Kruskal-Wallis test. The difference between samples treated with and without extra iron source or ascorbic acid and between heated and non-heated samples was checked with t-test or its non-parametric alternative, Wilcoxon test.

Assumptions, such as linearity and homoscedasticity were checked before correlation analysis and Pearson (*r*) or Kendall (*tau*) coefficients were applied for parametric and non-parametric analysis, respectively; confidence level was set at 95% ($p<0.05$) in both cases.

All statistical analyses and the data graphical visualization were performed using R (version 3.5.1; https://www.r-project.org/) and the RStudio Desktop software (http://www.rstudio.com/). Packages *agricolae* (Mendiburu 2020) and *ggplot2* (Wickham 2016) were applied to run Tukey's test and to prepare the graphs, respectively. Unless specified, values throughout the studies are presented as means of three replicates ± SD.

Image processing (cropping, scaling, and editing) was also carried out in R with *magick* package (Ooms 2021).

5.6 Eco-physiological characterisation of glyphosate-tolerant bacteria

5.6.1 pH spectrum testing

The effect of pH on the growth of glyphosate-tolerant bacteria was studied using YEG medium amended with Britton-Robinson buffer (Britton and Robinson 1931, Section 5.2). One hundred ml of solution I was mixed with solution II (42.5, 47.5, 52.5, 57.5, 60.0, 62.5 and 67.5 ml) to obtain pH values of 6.09, 6.59, 7.00, 7.54, 7.96, 8.36 and 8.95, respectively. The overnight bacterial strains pre-grown in 5 ml PDB were inoculated into 15-ml test tubes containing 5 ml YEG medium with the desired pH value at 10^5 CFU/ml. The OD of each culture was measured after 5 days of incubation at 620 nm (OD_{620}, SPECTROstar Nano microplate reader, BMG Labtech, Offenburg, Germany). Both solutions and YEG were prepared at two-times concentrations and mixed in equal proportions.

5.6.2 Heavy metal tolerance assay

The influence of 10 heavy metals (in the form of salts $AlCl_3 \cdot 6H_2O$, $CdSO_4 \cdot 5H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, $HgCl_2$, $K_2Cr_2O_7$, $MnSO_4 \cdot H_2O$, $NiSO_4 \cdot 7H_2O$, $Pb(NO_3)_2$, and $ZnSO_4 \cdot H_2O$) was studied on bacterial growth at 0.1, 0.5 and 1.0 mM concentrations of the metal ions according to the method described by Vörös et al. (2019). Overnight-grown liquid cultures of bacterial isolates were inoculated into 15-ml test tubes containing 5 ml PDB amended with the individual heavy metals at the initial concentration of 10^5 CFU/ml (as described above). Inoculated PDB containing no metal compound and non-inoculated PDB amended with the corresponding heavy metals were applied as positive and negative controls, respectively. The cell density (OD_{620}) was measured after a five-day incubation.

5.6.3 Pesticide sensitivity test

The technique reported by Vörös et al. (2019) was applied to examine the influence of different pesticides (Sigma-Aldrich, Budapest, Hungary) on bacterial growth with minor modifications. Eighteen herbicides, bensulfuron-methyl, chlorotoluron, chlorpropham, chlorsulfuron, cinosulfuron, diuron, dimethachlor, fenuron, ethoxysulfuron, glyphosate (AK Scientific, Union City, CA, USA), commercial formulation of glyphosate (*Glialka Star*), isoproturon, linuron, 2-methyl-4-chlorophenoxyacetic acid (MCPA), primisulfuron-methyl, propham, triasulfuron and 2,4-dichlorophenoxyacetic acid (2,4-D); fourteen fungicides captan, carbendazim, carboxin, fenarimol, flutriafol, imazalil, mancozeb, maneb, penconazole, tebuconazole, thiabendazole, thiram, thiophanate-methyl, and zineb, as well as the insecticide, diflubenzuron were added to 100 µl PDB in 96-well microtiter plates at 25, 12.5 and 6.25 µg/ml concentrations using serial dilution (broth microdilution assay). All pesticides were dissolved in 96% ethanol at 0.25 mg/ml (based on Pesticide Properties DataBase (PPDB) data of solubility), except for glyphosate, which was dissolved in distilled water. Overnight cultures pre-grown in 5 ml PDB were used to prepare bacterial suspensions at 10^5 CFU/ml in 5 ml physiological saline solution, and the wells of a microtiter plate were inoculated from the suspensions to a final concentration of 2×10^4 CFU/ml. Inoculated PDB containing no pesticide and non-inoculated PDB amended with the corresponding pesticides served as positive and negative controls, respectively. Due to differences between the growth rate of *P. resinovorans* and *E. adhaerens*, cell density (OD_{620}) was determined on the 2nd and the 5th day of incubation, accordingly.

5.6.4 Salinity tolerance assay

Broth microdilution method in 96-well microtiter plates was applied in the salinity tolerance assay and carried out according to Patel et al. (2017). The isolates were inoculated in 100 µl PDB amended with different concentrations of NaCl (100, 50, 25, 12.5, 6.3, 3.1, and 1.6 g/l) at 2×10^4 CFU/ml, as described above. Inoculated PDB containing no NaCl and non-inoculated PDB amended with NaCl at the appropriate concentrations served as the positive and negative controls, respectively. Cell density (OD_{620}) was determined after 2 or 5 days of incubation depending on the species.

5.6.5 Drought tolerance test

To simulate drought stress conditions during bacterial growth, polyethylene glycol (PEG) 6000 was added to liquid medium. The assay was carried out according to the method described by Devi et al. (2018) with modifications. Serial dilution of PEG was prepared in 100 µl PDB medium in 96-well microtiter plates to obtain concentrations of 250, 125, 62.5, 31.3, 15.6, 7.8, and 3.9 g/l (equivalent to osmotic potential values of −0.74, −0.22, −0.07, −0.03, −0.01, −0.005, and −0.002 MPa, respectively; Michel and Kaufmann 1973) and the inoculation was performed as described above. Inoculated PDB without PEG was used as positive control, while non-inoculated PDB containing PEG at the corresponding concentrations served as negative controls. Cell density (OD_{620}) was measured after 2 or 5 days of incubation depending on the species.

5.6.6 Statistical analysis

Assumptions for parametric tests as normal distribution of the residuals and homoscedasticity (similar variances in all groups) were checked before applying statistical test. The effect of the studied abiotic factors (salinity, drought, heavy metals, and pesticides) on bacterial growth was analysed by one-way ANOVA. Each significant ANOVA result ($p < 0.05$ level) was followed by post-hoc pair-wise comparisons (Tukey's test). The variants not satisfying the mentioned assumptions for parametric tests were analysed using Kruskal-Wallis test (non-parametric alternative for ANOVA). All statistical analyses and graphical presentations of the data were performed using R (version 3.5.1; https://www.r-project.org/) and the RStudio Desktop software (http://www.rstudio.com/). Packages *ggplot2* (Wickham 2016) and *patchwork* (Pedersen 2020) were applied for data visualization, package *agricolae* (Mendiburu 2020) was used for multiple comparisons of treatments (Tukey's HSD test). Unless specified, values throughout the study are means of three replicates ± SD.

5.7 Whole genome sequence analysis of selected glyphosate-tolerant bacteria

Based on the results of the previous studies, the 3 most prosperous glyphosate-tolerant isolates were selected for whole genome sequence analysis. The beneficial properties of the strains are listed in Table 8.

Table 8. Beneficial properties of glyphosate-tolerant isolates subjected to whole genome sequence analysis

Beneficial characteristics	**Glyphosate-tolerant bacteria**		
	***E. adhaerens* SZMC 25856**	***P. resinovorans* (genotype I) SZMC 25872**	***P. resinovorans* (genotype II) SZMC 25875**
Plant-growth promotion			

Promotion of tomato growth	✓	-	✓
IAA synthesis	✓	✓	✓
Ammonia synthesis	✓	✓	✓
Siderophore production	✓	✓	✓
Biocontrol potential			
Suppression of *A. tumefaciens*	-	✓	✓
Suppression of *A. vitis*	-	✓	✓
Eco-physiological properties			
Salinity tolerance	✓	✓	✓
Drought tolerance	✓	✓	✓
Pesticide tolerance	✓	✓	✓
Heavy metal tolerance	✓	✓	✓

Genomic DNA was extracted from the examined glyphosate-tolerant isolates using E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek, Norcross, GA, USA), following the instructions provided by the manufacturer. Next-generation sequencing and bioinformatical analysis was performed by the aid of an external service (SeqOmics Biotechnology Ltd., Hungary). The isolated genomic DNA was used for library preparation, followed by NGS as described in Section 5.1. Primary data analysis (base-calling) was carried out with Bbcl2fastq^ software (v2.17.1.14, Illumina). Reads were quality and length trimmed in CLC Genomics Workbench Tool 9.5.1 using an error probability of 0.05 (Q13) and a minimum length of 50 nucleotides as threshold. The length of the reads was 150 nt, and their total amount was 17,921,146; 18,263,522, and 16,422,194 in the case of the *E. adhaerens* SZMC 25856, *P. resinovorans* SZMC 25872, and *P. resinovorans* SZMC 25875, respectively. Mapping of the reads to the reference strains (Table 9) was carried out using the latest version of CLC Genomics Workbench 21. The analysis was carried out with Illumina mapper included in the software („Map to reference sequence" function) while the functional annotation of the extracted consensus sequences was performed with myRAST software.

Table 9. Reference strains used for the whole genome sequence analysis of glyphosate-tolerant bacteria

Test strains	Reference strains	GenBank ID
***E. adhaerens* SZMC 25856**	*E. adhaerens* Casida A	NZ_CP015880.1 (chromosome, 4,071,555 nt) NZ_CP015881.1 (plasmid A, 1,737,170 nt) NZ_CP015882.1 (plasmid B, 1,459,479 nt)
***P. resinovorans* SZMC 25872**	*P. resinovorans* NBRC 106553	NC_021499.1 (chromosome, 6,285,863 nt) NC_021506.1 (plasmid, 198,965 nt)
***P. resinovorans* SZMC 25875**		

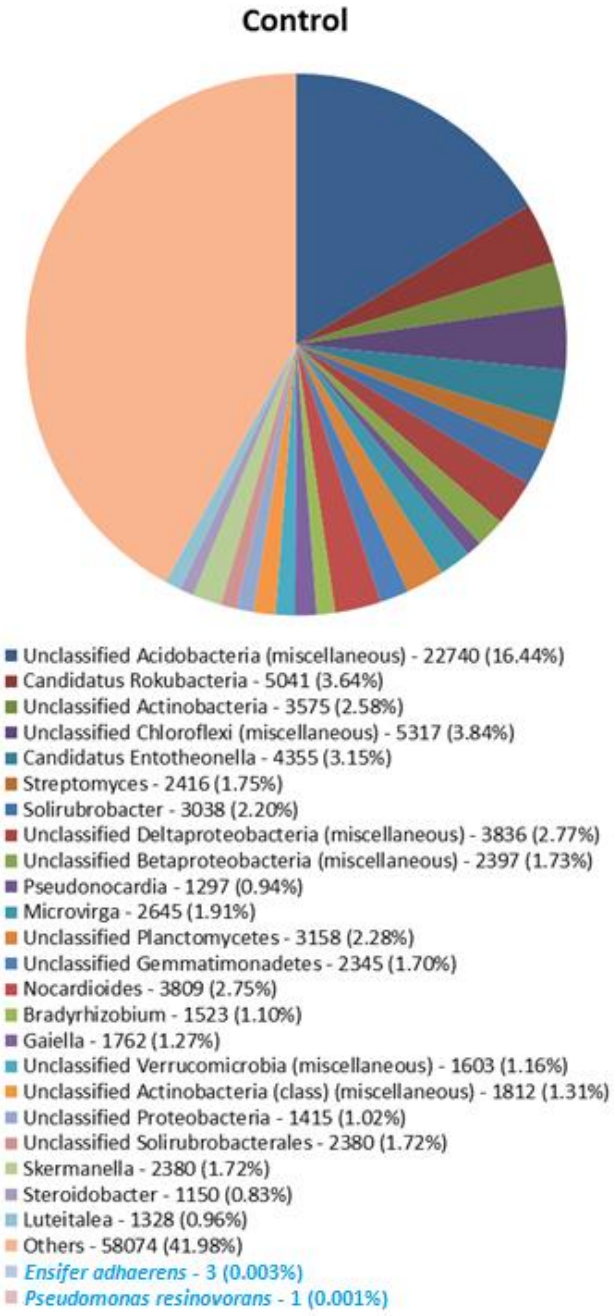
6 RESULTS AND DISCUSSION

6.1 Soil metagenome analysis

Based on the metagenome analysis, the presence of glyphosate seems to have a substantial influence on the composition of soil-inhabiting microbial populations. The most obvious difference was the high abundance of *Acidobacteria* in the control sample, however, they could not be detected in the glyphosate-treated soil. The members of the genus *Pseudonocardia* were not found in glyphosate-treated soil, while they were present in the control sample. However, glyphosate application seems to promote the presence of several bacteria, such as *Chloroflexi*, *Betaproteobacteria*, and *Nocardioides*, moreover, the genus *Gaiella* was only detected in glyphosate-treated soil sample. The occurrence of several bacterial groups including *Candidatus*, *Rokubacteria*, *Actinobacteria*, *Streptomyces*, *Solirubrobacter*, and *Microvirga* seems not to be affected by glyphosate, as they were detected in similar proportions in both the control and the glyphosate-treated samples. (Figure 3 A, B).

Based on the literature data about the influence of glyphosate on soil microorganisms, it seems to be species-dependent, as both growth promotion and suppression have been found in the case of different species. The DNA-based analysis of the rhizospheric microbiome of oat (*Avena sativa*) treated with glyphosate herbicides revealed no differences in diversity, but influence was detected on certain bacterial taxa. *Gaiella* and *Mesorhizobium* sequences were found in a lower and higher relative abundance in rhizosphere treated with glyphosate, respectively (Allegrini et al. 2019). Schlatter et al. (2017) reported that the 20-year-long repeated glyphosate application resulted in only 2-5% alteration in the bacterial community of wheat, and only less than 1% of all taxa was influenced by glyphosate, moreover, the relative abundance showed an increasing tendency. The authors proposed that the stimulatory effect on the bacterial groups was the consequence of the increase in the amount of dying roots after glyphosate usage, serving as a nutrient source. Furthermore, certain strains of different bacterial species, such as *B. cereus*, *Comamonas odontotermitis*, and *Pseudomonas* sp. have been reported to be able to utilise glyphosate either as the sole carbon or the phosphorus source (Jacob et al. 1988, Fan et al. 2012, Firdous et al. 2017). However, the detrimental effect of glyphosate on different soil-inhabiting organisms including bacteria, fungi, and earthworms has also been widely reported (Huber et al. 2005, Gaupp-Berghausen et al. 2015, Druille et al. 2016). The exposure to glyphosate has led to changes in the gut microbiota of different organisms, such as bees and cows (Krüger et al. 2013, Van Bruggen et al. 2018, Motta et al. 2018, Rueda-Ruzafa et al. 2019). Therefore, as glyphosate has been the most popular, globally-used herbicide since 1974 (Duke and Powles 2008), it is apparent that a broad range of agricultural soils have become polluted by glyphosate and its intermediates, which might have inhibitory effect on microorganisms, the beneficial strains to be introduced into agricultural soil need to be tested for their glyphosate tolerance in advance.

A


Control
Unclassified Acidobacteria (miscellaneous) - 22740 (16.44%)
Candidatus Rokubacteria - 5041 (3.64%)
Unclassified Actinobacteria - 3575 (2.58%)
Unclassified Chloroflexi (miscellaneous) - 5317 (3.84%)
Candidatus Entotheonella - 4355 (3.15%)
Streptomyces - 2416 (1.75%)
Solirubrobacter - 3038 (2.20%)
Unclassified Deltaproteobacteria (miscellaneous) - 3836 (2.77%)
Unclassified Betaproteobacteria (miscellaneous) - 2397 (1.73%)
Pseudonocardia - 1297 (0.94%)
Microvirga - 2645 (1.91%)
Unclassified Planctomycetes - 3158 (2.28%)
Unclassified Gemmatimonadetes - 2345 (1.70%)
Nocardioides - 3809 (2.75%)
Bradyrhizobium - 1523 (1.10%)
Gaiella - 1762 (1.27%)
Unclassified Verrucomicrobia (miscellaneous) - 1603 (1.16%)
Unclassified Actinobacteria (class) (miscellaneous) - 1812 (1.31%)
Unclassified Proteobacteria - 1415 (1.02%)
Unclassified Solirubrobacterales - 2380 (1.72%)
Skermanella - 2380 (1.72%)
Steroidobacter - 1150 (0.83%)
Luteitalea - 1328 (0.96%)
Others - 58074 (41.98%)
Ensifer adhaerens - 3 (0.003%)
Pseudomonas resinovorans - 1 (0.001%)

B

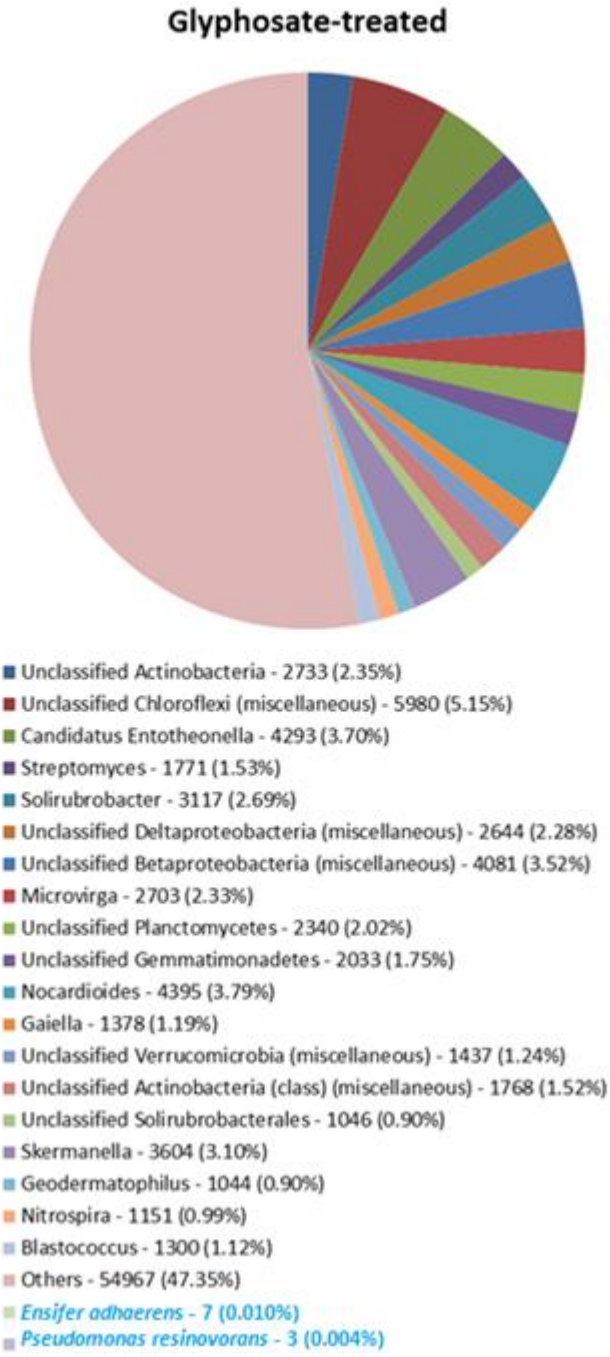


Figure 3. Composition of the bacterial populations of the control (A) and glyphosate-treated (B) soil samples

6.2 Isolation and identification of glyphosate-tolerant bacteria

A total of 30 bacterial strains were isolated from glyphosate-treated soil, and their growth was evaluated on SMM supplied with 1 g/l glyphosate as the sole carbon and nitrogen source. Species identification was performed in the case of 10 selected strains showing the most intensive growth after 7-day incubation, which possessed different morphology. Based on the sequence analysis of a fragment of the 16S rRNA gene, two isolates were identified as *E. adhaerens* (syn.: *Sinorhizobium morelense*), one strain as *Ochrobactrum anthropi*, and the remaining seven isolates were found to belong to the genus *Pseudomonas*, however, precise diagnosis could not be given. In these cases, sequences of the RNA polymerase subunit beta (*rpoB*) gene were also analysed, resulting in the identification of the seven strains as *P. resinovorans*, representing two different genotypes (I and II, involving 3 and 4 isolates, respectively) (Table 10, Figure 4). The metagenome analysis has revealed higher abundance of both *E. adhaerens* and *P. resinovorans* in the glyphosate-treated soil samples compared to the control specimen (Fig. 3A, B). However, their proportion among the detected bacterial species was still very low, confirming the need for the application of enrichment techniques for strain isolation (Jacquiod et al. 2013, Spini et al. 2018, Hii et al. 2020).

Table 10. Isolation and identification of glyphosate-tolerant bacteria

SZMC number	**Colony diameter (mm), *N*=4**	**Species identification***		
		Locus (gene)	**GenBank accession number**	**Species**
25848	4.00±0.00	*rpoB*	MT955645	*Pseudomonas resinovorans* genotype II
25851	5.75±0.50	*rpoB*	MT955646	*P. resinovorans*, genotype II
25853	2.25±0.50	16S rRNA	MT950352	*Ochrobactrum anthropi*
25856	2.25±0.50	16S rRNA	MT950353	*Ensifer adhaerens*
25859	4.00±0.00	*rpoB*	MT955647	*P. resinovorans*, genotype II
25863	5.00±0.00	*rpoB*	MT955648	*P. resinovorans*, genotype I
25870	5.25±0.50	*rpoB*	MT955649	*P. resinovorans*, genotype I
25871	2.00±0.00	16S rRNA	MT950357	*E. adhaerens*
25872	5.75±1.50	*rpoB*	MT955650	*P. resinovorans*, genotype I
25875	6.00±0.82	*rpoB*	MT955651	*P. resinovorans*, genotype II

*BLAST hits on January 25th, 2019

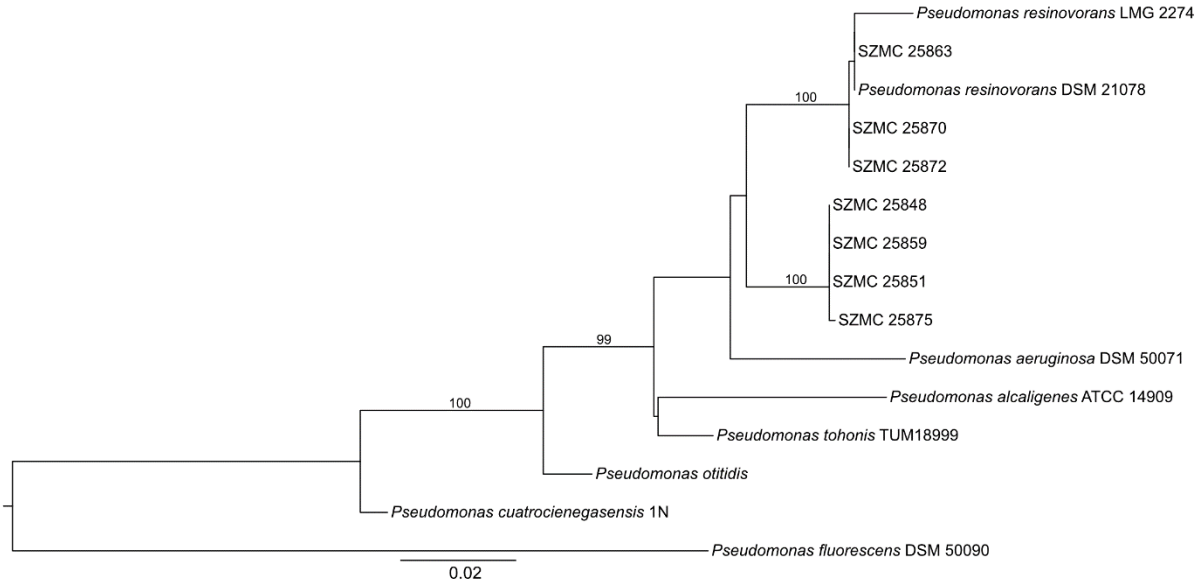


Figure 4. Phylogenetic tree of the *P. resinovorans* isolates, constructed using IQ-TREE v. 1.6.12 (Nguyen et al. 2015) with the K2P+G4 model for the 16S rRNA and HKY+F+G4 model for the *rpoB* dataset determined by the inbuilt ModelFinder tool (Kalyaanamoorthy et al. 2017). Statistical support of the best tree was calculated with Ultrafast bootstrap approximation (Hoang et al. 2018) with 5000 replicates. Bootstrap values above 95% - providing reliable results - are shown.

Since *O. anthropi* is reported as an opportunistic human pathogen (Ryan and Pembroke 2020) which limits remarkably its potential utilisation in agriculture, the isolate SZMC 25853 was excluded from the follow-up studies.

6.3 Examination of the plant growth-promoting potential of glyphosate-tolerant bacteria

6.3.1 Enhancement of tomato growth

Significant increase in the root length of tomato seedlings was induced by *E. adhaerens* SZMC 25856 and *P. resinovorans* SZMC 25875 compared to water and PDB controls, which resulted in higher total (seedling and root) length values (39.43% and 49.24%, as well as 32.23% and 41.53%, respectively, $p<0.05$). Beneficial influence of bacterial cultures on tomato growth was observed also in the case of *P. resinovorans* SZMC 25848 and SZMC 25872, which caused increased root length, while treatments with *P. resinovorans* SZMC 25870 and *E. adhaerens* SZMC 25871 resulted in higher root and total length values than control variants, but these values were not significantly different from the control data ($p>0.05$) (Figure 5). No significant effect on germination rate, fresh mass, vigor index as well as seedling length was detected ($p>0.05$).

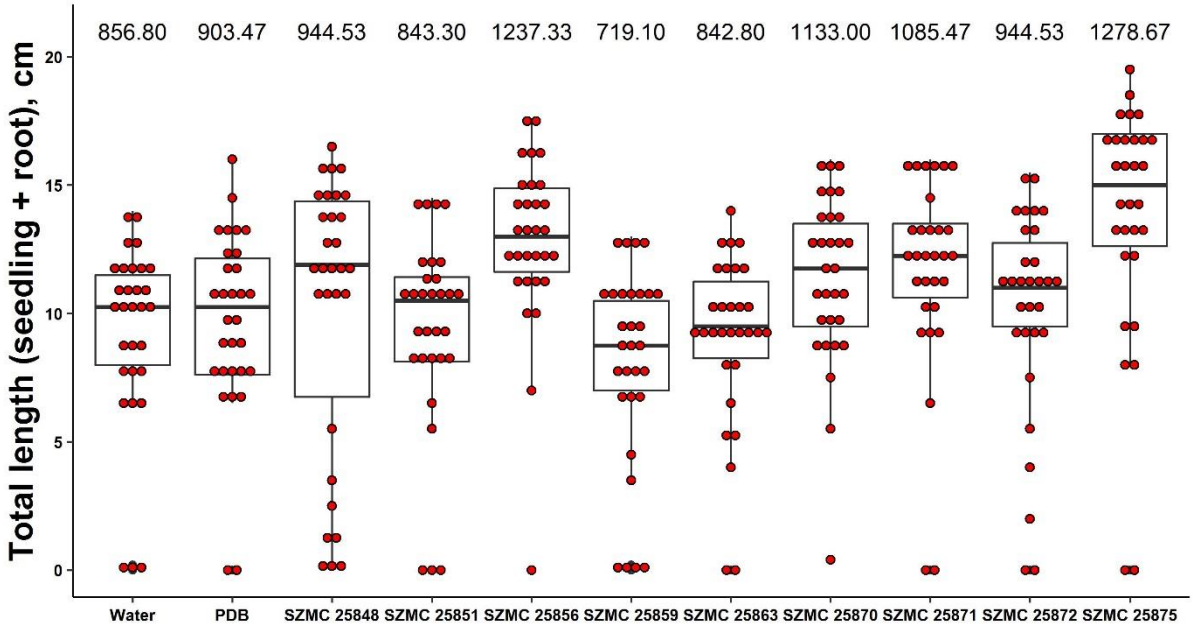


Figure 5. Enhancement of tomato growth by glyphosate-tolerant bacteria (Each dot represents the value of a single seed, *N* = 30. Non-germinated seeds are included with 0 values. Vigor index is indicated above each box.)

Considering the significant positive effect on tomato growth ($p<0.05$), two promising strains *E. adhaerens* SZMC 25856 and *P. resinovorans* (genotype II) SZMC 25875 were selected for further studies to examine their plant growth-promoting traits as well as their tolerance to different adverse abiotic factors (Section 6.5). *P. resinovorans* SZMC (genotype I) 25872 strain, showing biocontrol potential against *A. tumefaciens* (Section 6.4) was also included in the studies of plant growth promotion and eco-physiological characterisation.

Zhou et al. (2013) reported that the treatment of soybean seeds with *E. adhaerens* TMX-23 under salinity stress did not cause increase in the total length of the plants, yet improved vigour index by enhancing the germination rate. The application of the *P. resinovorans* AF22 strain on rice plants resulted in a significant increase in shoot and root dry weight but did not increase shoot height or root length (Habibi et al. 2019). Different *P. resinovorans* isolates might play additional roles in improving soil quality, as Matsushita et al. (2018) have reported about the ability of the *P. resinovorans* MO-1 strain that can oxidize Mn (II) and produce Mn (III, IV) oxide minerals, which take part in different biological processes and functions, such as photosynthesis, or protection against UV radiation, ROS and predation (Geszvain et al. 2012). The application of *P. mendocina in vivo* resulted in enhanced soil aggregate stability and significant improvement of the dehydrogenase and phosphatase activities by 21 and 89%, respectively (Kohler et al. 2006).

Neither *E. adhaerens*, nor *P. resinovorans* are well-characterised in the literature, but the abovementioned reports together with our findings propose these strains to possess yet unexplored potential for plant growth-promotion and protection from plant pathogens.

6.3.2 Indole-3-acetic-acid biosynthesis

Screening on PDA plates amended with L-tryptophan as the precursor of IAA revealed the IAA-synthesising ability of *E. adhaerens* SZMC 25856 as well as *P. resinovorans* (genotype I) SZMC 25872 isolate (Figure 6). Clearly visible pink-coloured zones were formed around the holes inoculated with these strains after the addition of Salkowski reagent,

while no or weak pink colour appeared around the colonies of *P. resinovorans* (genotype II) SZMC 25875.

Figure 6. Plate assays for screening of IAA-synthesising strains: **A** – strains 1, 4, and 9 (laboratory codes for *P. resinovorans* SZMC 25848, *P. resinovorans* SZMC 25851, and *E. adhaerens* SZMC 25856); **B** – strains 12, 16, and 23 (*P. resinovorans* SZMC 25859, *P. resinovorans* SZMC 25863, and *P. resinovorans* SZMC 25870); **C** – strains 24, 25, and 28 (*E. adhaerens* SZMC 25871, *P. resinovorans* SZMC 25872, and *P. resinovorans* SZMC 25875), respectively.

The subsequent spectrophotometric analysis based on the reaction of the culture supernatant obtained from PDB with and without L-tryptophan with Salkowski reagent revealed no visual reaction in supernatant from PDB without tryptophan which were confirmed by the same values of OD_{530} between non-inoculated and inoculated samples (Table 11). An increase in OD_{530} due to the reaction between Salkowski reagent and IAA produced by the strains was observed in the supernatant of PDB amended with L-tryptophan. Comparing them to non-inoculated control values (OD_{530} was 0.050±0.00 and 0.117±0.01 in non-inoculated PDB without and with tryptophan, respectively), a significant increase in OD_{530} ($p<0.05$) was observed in the case of the same strains, which showed intense pink colour around the colonies in the plate assays of *E. adhaerens* SZMC 25856 and *P. resinovorans* (genotype I) SZMC 25872, (Figure 6), and these isolates were involved in further HPLC-MS analysis for IAA quantification. A slight increase in OD_{530} observed on *P. resinovorans* (genotype II) SZMC 25875 was not significantly higher ($p>0.05$) than the non-inoculated control values, therefore, these strains were not selected for the IAA determination by HPLC-MS.

According to the results of HPLC-MS analysis, the amount of IAA in extracts from PDB supplied with tryptophan was detected as 0.28 and 0.23 µg/ml in the case of *E. adhaerens* SZMC 25856 and *P. resinovorans* (genotype I) SZMC 25872, respectively (Table 11). Interestingly, both strains were also able to synthesize minor quantities of IAA (0.005-0.027 µg/ml) in PDB without L-tryptophan.

In our studies, IAA production of *E. adhaerens* SZMC 25856 was detected as 0.28 and 0.005 µg/ml in PDB amended with and without L-tryptophan, respectively. The IAA-synthesising ability of *E. adhaerens* TMX-23 and *E. adhaerens* OS3 has been reported previously, and IAA concentration was found as 8.2 and 30.6 µg/ml, respectively (Zhou et al. 2013, Oves et al. 2017), however, the mentioned data were obtained by spectrophotometry. The colourimetric analysis of Mesa et al. (2017) also revealed the synthesis of 30.01 µg/ml IAA by the *E. adhaerens* 91R. In our experiments, neither plate, nor colourimetric assays revealed IAA production of *P. resinovorans* (genotype II) SZMC 25875, therefore, only *P. resinovorans* (genotype I) SZMC 25872 was subjected to HPLC analysis, which confirmed its ability to produce 0.23 µg/ml IAA. According to the results of the spectrophotometric assays performed by Habibi et al. (2019), *P. resinovorans* AF22 was also able to produce 3.9 µg/ml IAA in liquid culture.

To the best of our knowledge, this is the first report on the IAA production of *E. adhaerens* and *P. resinovorans* strains quantified by HPLC analysis (Table 11). Certain amount of IAA was detected also in PDB without the addition of the IAA precursor L-tryptophan, which might be explained with the composition of the growth medium. As PDB is a plant (potato)-based medium, it may contain naturally produced tryptophan, which might have been used by the bacteria for the synthesis of IAA. However, recent findings suggest that besides the five different pathways involved in the tryptophan-dependent IAA synthesis of Gram-negative bacteria, IAA production might occur in the absence of tryptophan in both bacteria and plants (Keswani et al. 2020). Prinsen et al. (1993) firstly reported about tryptophan-independent IAA biosynthesis by *A. brasilense*, and it was confirmed by recent findings, in which IAA production through a tryptophan-independent pathway by *Arthrobacter pascens* ZZ21 was also proposed (Li et al. 2018). Furthermore, *B. cereus* So3II and *B. subtilis* Mt3b were also reported to produce IAA in both the presence and absence of tryptophan (Wagi and Ahmed 2019). Remarkably high amounts of IAA were detected in the tryptophan-free culture supernatants of the endophytic fungal isolates *Alternaria* sp. SZMC 26651 and *Didymella* sp. SZMC 26655 (Turbat et al. 2020), while another endophytic fungal strain, *Aureobasidium* sp. BSS6 was also able to synthesise IAA via both tryptophan-dependent and independent pathways (Khan et al. 2016).

IAA is known as a phytohormone being crucial in nearly all aspects of plant growth. IAA-synthesising species proved to promote the growth of a variety of agricultural crops even under abiotic stress factors (Duca et al. 2014, Etesami and Maheshwari 2018, Brilli et al. 2019). The ability of *E. adhaerens* SZMC 25856 and *P. resinovorans* SZMC 25872 to produce IAA may be suggested as an important plant growth-promoting characteristic of these strains.

Table 11. Plant growth-promoting traits of glyphosate-tolerant bacteria

Isolate	IAA production					Siderophore production			NH_3 production, mg/ml	Phosphorus solubilisation
	Development of pink colour (plate assays)	Spectrophotometric assay (OD_{530})		HPLC measurements µg/ml		Diameter, mm		%		
		Try+	Try-	Try+	Try-	Colony	Halo zone			
E. adhaerens SZMC 25856	+	0.337±0.03	0.050±0.00	0.28±0.03	0.005±0.00	2.83±1.89	5.67±1.53	24.59±12.18	0.11±0.04	-
P. resinovorans (genotype I) SZMC 25872	+	0.217±0.04	0.080±0.00	0.23±0.05	0.027±0.01	24.33±2.52	35.00±1.00	21.66±1.39	0.13±0.02	-
P. resinovorans (genotype II) SZMC 25875	-	0.140±0.01	0.063±0.01	nd	nd	24.00±3.00	24.00±3.00	13.73±4.11	0.14±0.03	-

nd: no data available

6.3.3 Siderophore production

The most remarkable orange halo zones, indicating siderophore production on CAS agar plate containing peptone and yeast extract as carbon sources were produced by *P. resinovorans* (genotype I) SZMC 25872 (35.00 mm) (Table 11, Figure 7). Despite the slower growth compared to *P. resinovorans* strains, *E. adhaerens* SZMC 25856 was also able to produce halo zone of 5.67 mm, while no orange halo zone was produced by *P. resinovorans* (genotype II) SZMC 25875.

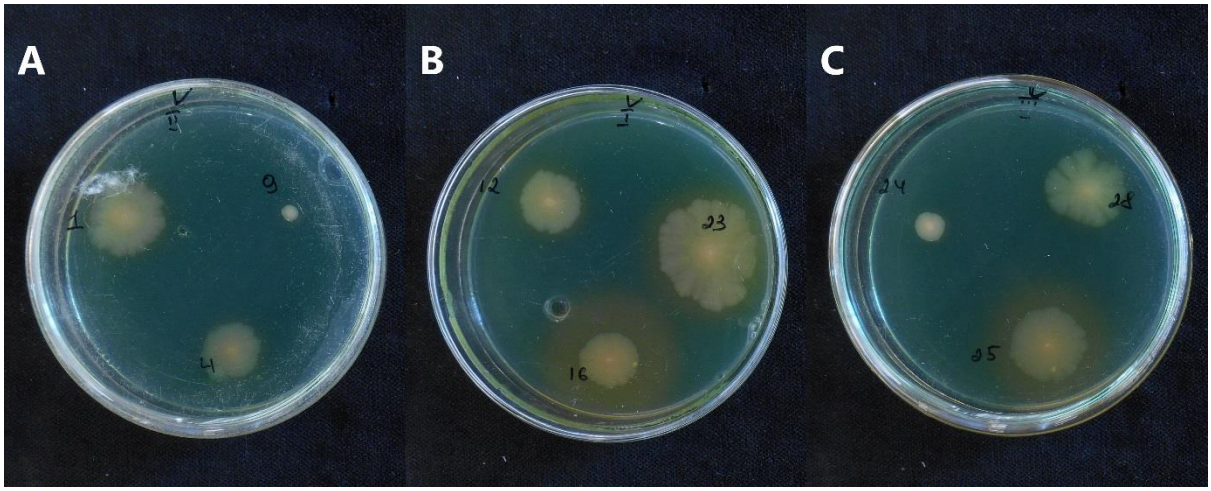


Figure 7. Plate assays with blue agar CAS dye for the screening of siderophore-producing strains: **A** – strains 1, 4, and 9 (laboratory codes for *P. resinovorans* SZMC 25848, *P. resinovorans* SZMC 25851, and *E. adhaerens* SZMC 25856); **B** – strains 12, 16, and 23 (*P. resinovorans* SZMC 25859, *P. resinovorans* SZMC 25863, and *P. resinovorans* SZMC 25870); **C** – strains 24, 25, and 28 (*E. adhaerens* SZMC 25871, *P. resinovorans* SZMC 25872, and *P. resinovorans* SZMC 25875), respectively.

In contrast, spectrophotometric assays revealed the highest siderophore-producing ability of *E. adhaerens* SZMC 25856 (24.59%), *P. resinovorans* (genotype I) SZMC 25872 (21.66%) and *P. resinovorans* (genotype II) SZMC 25875 (13.73%) in liquid medium containing succinate as carbon source. The follow-up studies revealed a crucial influence of growth medium on siderophore production of all the above-mentioned strains (Subsection 6.4.6). Among 7 various media (PDB, LB, YEG, and MM supplied with glucose, L-alanine, succinic acid, and sodium pyruvate), the highest siderophore yields were detected in MM amended with succinic acid, followed by MM with L-alanine, while no siderophore production was found in PDB, LB, YEG, and MM supplied with glucose.

The *E. adhaerens* TMX-23 grown in medium supplied with mannitol and glutamine was characterised as a catechol-type siderophore-producing strain, and synthesized salicylic acid and dihydrobenzoic acid at 6.9 and 0.48 µg/ml concentrations, respectively (Zhou et al. 2013). Another *E. adhaerens* isolate, OS3 was also proved to be capable of siderophore production in both solid and liquid cultures, and its halo zone on CAS agar plate was 13.4 mm, while the concentrations of salicylic acid and dihydrobenzoic acid (produced in the same medium that was applied for *E. adhaerens* TMX-23) were 20.6 and 22.7 µg/ml, accordingly (Oves et al. 2017). In addition, *E. adhaerens* Sx1 was also reported to produce siderophores on CAS agar plates (Mesa et al. 2017). The siderophore-producing ability of different *Pseudomonas* species (Pahari et al. 2017), including *P. mendocina* (Kohler et al. 2006), *P. fulva* (Munir et al. 2019) and *P. chlororaphis* (Sharma and Johri 2003) has been reported, however, Habibi et al. (2019) observed no siderophore production by *P. resinovorans* AF22 on solid medium. Our *P. resinovorans* isolates of both genotypes were able to produce

siderophores, but differences were found between genotypes. In contrast to *E. adhaerens* SZMC 25856 and *P. resinovorans* (genotype I) SZMC 25872, *P. resinovorans* (genotype II) SZMC 25875 isolate did not form halo zones on CAS agar, however, siderophore production of all isolates has been detected in liquid standard succinate medium (Table 11). Similarly to our findings, Alexander and Zuberer (1991) reported about *P. putida* P4 and *E. herbicola* S47 that they did not develop halo zones on CAS agar plates, but still produced siderophores in liquid medium. The composition of the culture medium may affect siderophore production significantly. As it was mentioned above, during the biocontrol studies (Subsection 6.4.6), remarkable siderophore production was observed for all the 3 examined strains in iron-free MM containing succinic acid, L-alanine, and sodium pyruvate as carbon source, while siderophores could not be detected in iron-repleted media, such as PDB, YEG, and LB. Payne (1994) has stated that the use of succinate instead of glucose as carbon source can result in enhanced siderophore production. Ghosh et al. (2015) evaluated the effect of different media on siderophore production of *P. aeruginosa* and found that malt extract induced the highest production rate (80.50%). Media containing either casamino acids or succinic acid as the sole carbon source supported siderophore production, while no siderophores were detected in the medium containing peptone and beef extract. Statistical-based approach (Plackett–Burman design and response surface methodology) applied to evaluate the effect of 7 factors (concentration of K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$ and succinic acid, as well as pH and temperature levels) on the siderophore production of *P. aeruginosa* RZS9 revealed that succinic acid, pH, and temperature had significant influence on siderophore synthesis. The optimal conditions were found to be 4.9 g/l succinic acid, pH 7.08, and the temperature of 27.80 °C, resulting in the highest degree of siderophore production (68.41%) (Shaikh et al. 2016).

Based on the quantitative data obtained from different iron-free media in this test and the assays of the biocontrol activity, all the 3 tested glyphosate-tolerant bacterial isolates have the ability to produce siderophores, which is suggested to contribute to their plant-growth promotion potential.

6.3.4 Phosphorus solubilisation

The glyphosate-tolerant bacterial strains were grown on PM and MVM agar plates for 2 weeks, but no halo zone indicating the solubilisation of $Ca_3(PO_4)_2$ was developed by any isolate (Table 11). These results suggest that none of the studied strains possess phosphorus solubilising properties.

The solubilisation of inorganic insoluble phosphate can also play role in plant growth-promoting effect of certain soil microorganisms (Sharma et al. 2013). In our experiments, neither *E. adhaerens*, nor *P. resinovorans* isolates showed phosphate-solubilising activity on solid PM medium (Table 11). Likewise, the lack of phosphate solubilisation of *E. adhaerens* TMX-23 on PM agar medium was documented by Zhou et al. (2013). In contrast, Oves et al. (2017) reported *E. adhaerens* OS3 to have phosphate-solubilising activity. Several species of the genus *Pseudomonas*, such as *P. simiae*, *P. koreensis* (Kumari et al. 2016) and *P. fulva* (Munir et al. 2019) are known to possess phosphate-solubilising ability, however, the solubilisation of phosphate by *P. resinovorans* has not been reported yet. Similarly to our results, in the study of Habibi et al. (2019), *P. resinovorans* AF22 was also shown to lack phosphate solubilising potential. Although, the P-solubilising ability of bacteria is a valuable feature for plant growth promotion, none of the glyphosate-tolerant bacteria were found to possess this trait in our studies.

6.3.5 Ammonia production

All strains were able to produce remarkable quantities of ammonia in PW, suggesting their potential for plant growth stimulation. Ammonia concentration fell in range of 0.11-0.14 mg/ml, and no substantial differences were found either among species or strains (Table 11).

The ammonia-producing traits of all tested strains were confirmed by the Nesslerization reaction. Our results are supported by the findings of Zhou et al. (2013) and Oves et al. (2017), in which *E. adhaerens* TMX-23 and OS3 were reported as ammonia-producing isolates. The ammonia-producing ability of *P. resinovorans* has not been reported yet, although, other strains belonging to the *Pseudomonas* genus have also been found to produce ammonia at different concentrations. The marine isolate *P. aeruginosa* BG produced 0.027 mg/ml ammonia in 5 ml PW after 13-day incubation (Goswami et al. 2015). The development of red colour in the culture broth of *P. aeruginosa* GS-33 was also considered to be the sign of ammonia production (Patil et al. 2016). Different, non-identified N-fixing strains were able to excrete 0.000018-0.002 mg/ml ammonia (Kayasth et al. 2014).

Ammonia production is considered to be another beneficial characteristic, which enables microbial strains to provide crops with additional nitrogen source (Kayasth et al. 2014, Patil et al. 2016), suggesting that *E. adhaerens* SZMC 25856, as well as the *P. resinovorans* strains of both genotypes (SZMC 25872 and SZMC 25875) may have further potential to enhance crop production.

6.4 Investigation into the biocontrol potential of glyphosate-tolerant bacteria

6.4.1 Anti-quorum sensing activity

QS inhibition of *S. marcescens* required remarkable amount of the supernatant: sufficient inhibition zones started forming on 200 and 300 µl on all glyphosate-tolerant strains (Figure 8). No inhibition was found in the case of *C. violaceum* SZMC 6269.

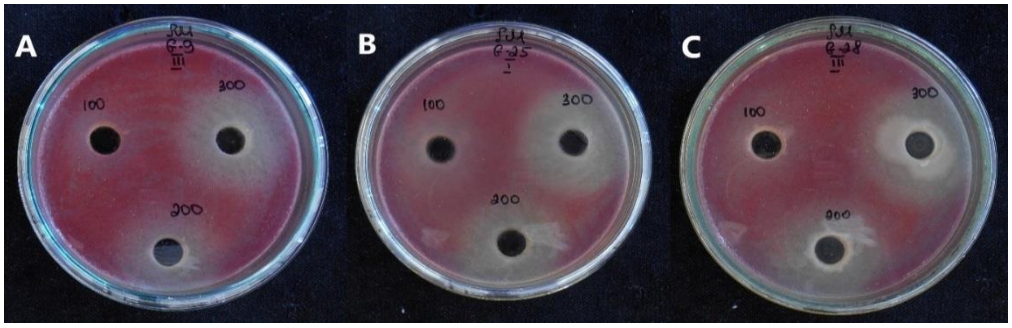


Figure 8. Quorum-sensing inhibition of *S. marcescens* SZMC 0567 by supernatant of glyphosate-tolerant strains: **A** – *E. adhaerens* SZMC 25856, **B** – *P. resinovorans* SZMC 25872, and **C** – *P. resinovorans* SZMC 25875.

QS system is specific for each species and several componds are reported as quorum-sensing inhibitors (Taga and Bassler 2003, Tay and Yew 2013). As QS is involved in many cellular mechanisms, such as antibiotic production, bioflim formation, plasmid conjugation, the inhibition of QS is considered as an effective strategy to control bacterial growh (Taga and Bassler 2003, Billot et al. 2020). Our findings suddested potential anti-quroum sensing activity of the supernatant of glyphosate-tolerant strains towards *S. marcescens*.

6.4.2 *In vitro* antagonistic activity towards plant pathogenic fungi and bacteria

Dual-culture tests revealed no inhibitory effect of glyphosate-tolerant strains on the growth of the observed plant pathogenic fungi (Table 5).

All isolates were tested for their potential antagonistic activity against plant pathogenic bacterial strains (Table 5). Among the tested pathogens, *P. resinovorans* strains were found to inhibit the growth of *A. tumefaciens* SZMC 21407. Therefore, the follow-up studies for biocontrol potential were carried out using 7 *A. tumefaciens* and 7 *A. vitis* strains, which were retrieved from the SZMC collection. All *P. resinovorans* strains of both genotypes were able to substantially inhibit 6 out of the 7 tested *A. tumefaciens* and *A. vitis* (Table 12) strains, however, only partial inhibition was observed in the case of *A. vitis*. No antagonistic activity was shown by the two *E. adhaerens* isolates. Single strains of *A. tumefaciens* SZMC 14554 and *A. vitis* SZMC 21784 were not inhibited by any of *P. resinovorans* isolates, while *A. tumefaciens* SZMC 14557 and *A. vitis* 21396 were found to be the most susceptible isolates, therefore, they were selected as model strains for further studies.

Among *P. resinovorans* strains, SZMC 25872 (genotype I) showed the most intensive inhibition of *A. tumefaciens* SZMC 14557 (Table 12) and was chosen for the detailed determination of its biocontrol potential and eco-physiological characterisation (Sections 6.4 and 6.5). In addition, being plant growth-promoting strains, *P. resinovorans* (genotype II) SZMC 25875 and *E. adhaerens* SZMC 25856 (Section 6.3) were also included in the follow-up biocontrol assays.

Table 12. Colony diameter (C, mm) and colony+inhibition zone diameter (IZ, mm) values of 7 *P. resinovorans* isolates on susceptible *A. tumefaciens* and *A. vitis* strains. The data are presented as means of 3 replicates±SD.

***P. resinovorans* isolates**	***A. tumefaciens* strains**											
	SZMC 14555		**SZMC 14556**		**SZMC 14557**		**SZMC 21395**		**SZMC 21407**		**SZMC 21783**	
	C	**IZ**	C	**IZ**	C	**IZ**	C	**IZ**	C	**IZ**	C	**IZ**
SZMC 25848	10.3±1.2	21.7±0.6	10.0±0.0	27.3±2.5	11.0±1.0	36.3±1.5	10.0±0.0	21.3±3.2	9.7±0.6	22.0±4.0	10.7±1.2	29.0±1.7
SZMC 25851	9.0±0.0	19.7±2.5	10.0±0.0	24.7±2.1	10.3±1.2	32.7±2.1	10.0±0.0	21.7±1.5	9.3±0.6	18.0±2.0	9.7±0.6	27.7±2.5
SZMC 25859	9.3±1.2	21.7±2.1	9.7±1.2	21.0±2.0	10.7±0.6	35.3±2.9	9.0±1.0	18.7±2.5	9.3±0.6	22.0±3.0	10.0±0.0	29.0±3.5
SZMC 25863	8.3±0.6	18.0±1.0	10.0±0.0	17.7±0.6	10.3±0.6	24.3±0.6	9.0±1.0	15.3±0.6	9.7±0.6	16.0±0.0	10.0±0.0	21.0±3.6
SZMC 25870	9.3±0.6	17.7±1.2	10.7±0.6	20.0±1.7	7.7±3.2	22.0±1.0	8.5±0.7	16.5±0.7*	9.3±0.6	16.7±0.6	10.0±1.0	20.3±2.1
SZMC 25872	9.3±0.6	19.0±2.0	9.7±0.6	17.0±2.0	10.7±0.6	24.7±1.5	9.0±1.0	16.0±1.0	9.0±1.4	14.5±0.7*	9.7±1.2	21.0±1.0
SZMC 25875	9.3±1.2	23.7±3.5	10.3±0.6	19.0±1.7	9.7±0.6	31.3±2.3	9.7±0.6	18.7±1.5	9.7±0.6	21.0±4.4	10.3±0.6	25.7±1.5
***P. resinovorans* isolates**	***A. vitis* strains**											
	SZMC 21396		**SZMC 21397**		**SZMC 21398**		**SZMC 21785**		**SZMC 21786**		**SZMC 21787**	
	C	**IZ**	C	**IZ**	C	**IZ**	C	**IZ**	C	**IZ**	C	**IZ**
SZMC 25848	9.7±0.6	34.7±2.9	10.0±1.0	16.0±0.0*	9.3±0.6	27.0±2.0	10.3±0.6	29.3±1.5	10.0±1.0	22.0**	10.0±0.0	35.0±1.4*
SZMC 25851	9.3±0.6	33.7±3.1	10.0±1.0	21.5±3.5*	9.7±0.6	29.0±2.0	9.7±0.6	29.3±5.0	10.3±0.6	22.0**	11.0±1.4	35.5±0.7*
SZMC 25859	9.7±0.6	34.7±5.5	10.3±0.6	0	10.0±1.0	27.0±7.2	10.3±0.6	31.0±1.0	9.3±0.6	26.0±7.1*	8.0±0.0	36.0**
SZMC 25863	9.3±0.6	28.0±1.0	10.0±0.0	0	10.7±0.6	21.7±1.5	10.0±1.0	22.3±2.5	10.3±1.2	23.0**	10.0±0.0	29.0±4.2*
SZMC 25870	10.3±0.6	28.3±1.5	11.0±1.0	0	9.7±1.2	18.0±2.0	11.0±1.0	23.7±3.1	9.3±0.6	22.0±1.4*	10.5±0.7	25.0±7.1*
SZMC 25872	10.0±0.0	28.3±0.6	11.0±1.0	20.5±2.1*	9.3±1.2	19.7±6.4	9.3±0.6	22.0±4.2*	9.7±0.6	22.0±0.0	9.5±0.7	26.0±1.4*
SZMC 25875	10.0±0.0	35.7±1.5	9.3±1.5	25.0±8.5*	10.3±0.6	30.7±1.5	11.7±3.1	27.5±0.7	10.3±0.6	26.3±2.5	11.0±0.0	26.0±2.8*

*the inhibition was observed on 2 replicates

**the inhibition was observed on 1 replicate

Both *P. resinovorans* strains (SZMC 25872 and SZMC 25875) showed sufficient inhibition activity towards the majority of the tested *A. tumefaciens* and *A. vitis* strains. To date, several bacterial species were reported to be capable of inhibiting *A. tumefaciens*. *P. fluorescens* B-4117 and Q8r1-96 suppressed the growth of both *A. tumefaciens* and *A. vitis* (Dandurishvili et al. 2010). The treatments of tomato plants with suspensions of both the vegetative cells and spores of *B. amyloliquefaciens* ssp. *plantarum* 32a resulted in a 79.1-87.5% reduction of the symptoms caused by *A. tumefaciens* C58 and B6 (Abdallah et al. 2018a). In the studies of Xie et al. (2021), phenylacetic acid and behenic acid produced by *B. megaterium* L2 were found to inhibit the growth of *A. tumefaciens*.

In general, *Pseudomonas* and *Bacillus* genera are considered to have the most promising species possessing antagonistic activity towards fungal and bacterial plant pathogens (Mnif and Ghribi 2015). Phenazine-type 1-hydroxyphenazine produced by *P. aeruginosa* SD12 inhibited *in vitro* various fungal pathogens, such as *Alternaria alternata, A. solani, Bipolaris australiensis, Colletotrichum acutatum, Curvularia andropogonis, F. oxysporum, F. moniliforme, P. aphanidermatum,* and *Rhizoctonia solani* (Dharni et al. 2012). *P. aeruginosa* LN strain was reported to possess biocontrol activity against *X. axonopodis* pv. *malvacearum*, pv. *phaseoli* and pv. *citri* (Spago et al. 2014). However, to the best of our knowledge, no strain of *P. resinovorans* species has been reported yet to possess the potential of controlling any pathogen. In contrast, *E. adhaerens* was previously reported as a predator of *Micrococcus luteus* (Casida 1982, Germida and Casida 1983), but in our studies, the SZMC 25856 strain did not show inhibitory effect on any of the tested pathogens.

Our findings can be summarised as *P. resinovorans* SZMC 25872 and SZMC 25875 can efficiently suppress the growth of *A. tumefaciens* strains, therefore, *P. resinovorans* can be proposed for the first time as a species with newly discovered biocontrol activity.

6.4.3 Carbon source utilisation tests

E. adhaerens SZMC 25856 and *P. resinovorans* (genotype II) SZMC 25875 as plant-growth-promoting strains (Section 6.3), as well as *P. resinovorans* (genotype I) SZMC 25872 as the most promising biocontrol isolate together with *A. tumefaciens* SZMC 14557 and *A. vitis* SZMC 21396, which proved to be the most susceptible pathogenic strains (Subsection 6.4.2) were selected for carbon source utilisation assays. All strains were grown in MM supplied with different compounds (2 g/l) individually to test their ability to utilise them as the sole carbon source (Table 13).

Table 13. The growth of bacterial strains in the presence of 80 different compounds as the sole carbon source

Carbon source	**Strains**				
	E. adhaerens **SZMC 25856**	***P. resinovorans*** **(genotype I) SZMC 25872**	***P. resinovorans*** **(genotype II) SZMC 25875**	***A. tumefaciens*** **SZMC 14557**	***A. vitis*** **SZMC 21396**
(-) quinic acid	-	-	-	-	-
2-keto-D-gluconic acid	-	**	***	***	-
adenosine	***	-	-	-	-
alpha-methyl-D-mannoside	-	-	-	-	-
ascorbic acid	-	-	-	-	-
beta-alanine	***	**	**	-	-
beta-methyl-D-galactoside (1-O-methyl-beta-D-galactopyranoside)	***	*	-	**	-
cellobiose	***	-	-	**	*
cis-aconitic acid	-	-	-	-	-
cytidine	***	*	*	-	-
cytosine	-	-	-	-	-
D-arabinose	***	*	-	**	-
dextran	**	*	-	*	-
D-fructose	***	**	-	***	**
D-galactose	***	-	*	**	**
D-glucuronic acid	***	***	-	**	-
D-glucosamine	***	*	-	**	-
D-glucose	***	**	***	**	**
dihydroxyacetone	***	-	-	-	-
DL-isocitric acid	-	***	***	-	-
D-lyxose	***	*	-	**	**
D-mannitol	***	-	-	***	**
D-mannose	***	-	-	**	**
D-xylose	***	**	-	**	**

ethanol	**	-	-	**	-
fumaric acid	-	-	-	-	-
galactitol	-	-	-	**	-
gallic acid	-	-	-	-	-
gamma-butyrolactone	**	-	-	-	-
gentisic acid	-	-	-	-	-
gluconic acid	**	-	***	-	-
glycerol	***	-	-	***	*
glycerol-l-monoacetate	***	-	**	***	**
glycine	-	**	*	-	-
i-erythritol	**	-	-	-	-
inosine	-	-	-	-	-
inulin	-	*	-	-	-
ketoisovaleric acid	**	*	**	*	-
lactose	***	-	-	***	-
L-alanine	***	**	-	**	*
L-arabinose	***	-	-	**	**
L-arginine	***	***	***	-	-
L-asparagine	***	***	***	***	-
L-citrulline	***	-	*	-	-
L-glutamic acid	***	***	***	**	**
L-glutamine	***	***	***	***	**
L-histidine	*	**	*	*	-
L-isoleucine	-	*	-	-	-
L-lactic acid (Na-DL-lactate)	***	***	***	**	-
L-lysine	**	**	**	-	-
L-malic acid	***	***	***	***	**
L-methionine	-	-	-	-	-
L-ornithine	***	***	*	-	-
L-proline	***	***	***	**	*
L-rhamnose	***	-	-	**	**

L-serine	***	-	-	**	-
L-sorbose	-	-	-	*	-
L-threonine	***	-	-	-	-
L-tryptophan	*	-	-	-	-
L-valine	-	**	**	-	-
maltose	***	-	-	***	***
melezitose	***	-	-	***	-
melibiose	***	-	-	***	**
myo-inositol	***	-	-	***	*
nicotinic acid	-	-	-	-	-
p-arbutin	***	-	-	***	**
protocatechuic acid (3,4-dixydroxybenzoic acid)	-	-	-	-	-
pyruvic acid (sodium pyruvate)	***	***	***	**	*
raffinose (raffinose pentahydrate)	***	-	-	***	**
ribitol (adonitol)	***	-	-	***	**
sorbitol	***	-	-	**	**
starch	**	***	**	**	**
succinic acid	-	***	***	***	***
sucrose	***	-	-	***	**
tannic acid	***	***	**	**	***
thymine	-	-	*	-	-
uridine	***	-	-	**	-
vanillin	-	-	-	*	-
xylan	*	*	*	*	-
xylitol	**	-	*	**	-
water (control)	-0.015	-0.001	-0.010	0.024	0.015

-: no growth (OD_{620}= **0.00-0.050, (based on the maximum of control water (0.024) multiplied by 2)**
*: **weak growth** (OD_{620}= **0.050-0.1**)
: **moderate growth (OD_{620}= **0.1-0.2**)
***: **strong growth** (OD_{620} **>0.2**)

E. adhaerens SZMC 25856 was able to utilise 45 of the 80 tested compounds (adenosine, beta-alanine, beta-methyl-D-galactoside, cellobiose, cytidine, D-arabinose, D-fructose, D-galactose, D-glucuronic acid, D-glucosamine, D-glucose, dihydroxyacetone, D-lyxose, D-mannitol, D-mannose, D-xylose, glycerol, glycerol-l-monoacetate, lactose, L-alanine, L-arabinose, L-arginine, L-asparagine, L-citrulline, L-glutamic acid, L-glutamine, L-lactic acid, L-malic acid, L-ornithine, L-proline, L-rhamnose, L-serine, L-threonine, maltose, melezitose, melibiose, myo-inositol, p-arbutin, sodium pyruvate, raffinose, ribitol, sorbitol, sucrose, tannic acid, uridine) (Table 13) with OD_{620} >0.2. The OD_{620} values in the case of 9 (dextran, ethanol, gamma-butyrolactone, gluconic acid, i-erythritol, ketoisovaleric acid, L-lysine, starch, xylitol) and 3 compounds (L-histidine, L-tryptophan, xylan) were 0.1-0.2 and 0.05-0.1, respectively. Twenty-three compounds ((-) quinic acid, 2-keto-D-gluconic acid, alpha-methyl-D-mannoside, ascorbic acid, cis-aconitic acid, cytosine, DL-isocitric acid, fumaric acid, galactitol, gallic acid, gentisic acid, glycine, inosine, inulin, L-isoleucine, L-methionine, L-sorbose, L-valine, nicotinic acid, protocatechuic acid, succinic acid, thymine, vanillin) could not be utilised as the sole carbon source.

P. resinovorans (genotype I) SZMC 25872 showed substantial growth (OD_{620} >0.2) in the presence of 14 compounds (D-glucuronic acid, DL-isocitric acid, L-arginine, L-asparagine, L-glutamic acid, L-glutamine, L-lactic acid, L-malic acid, L-ornithine, L-proline, sodium pyruvate, starch, succinic acid, tannic acid). OD_{620} values of 10 substances (2-keto-D-gluconic acid, beta-alanine, D-fructose, D-glucose, D-xylose, glycine, L-alanine, L-histidine, L-lysine, L-valine) fell in the range 0.1-0.2, and low growth was observed (OD_{620} = 0.05-0.1) in the case of 10 compounds (beta-methyl-D-galactoside, cytidine, D-arabinose, dextran, D-glucosamine, D-lyxose, inulin, ketoisovaleric acid, L-isoleucine, xylan). The strain was not able to utilise 46 substances ((-) quinic acid, adenosine, alpha-methyl-D-mannoside, ascorbic acid, cellobiose, cis-aconitic acid, cytosine, D-galactose, dihydroxyacetone, D-mannitol, D-mannose, ethanol, fumaric acid, galactitol, gallic acid, gamma-butyrolactone, gentisic acid, gluconic acid, glycerol, glycerol-l-monoacetate, i-erythritol, inosine, lactose, L-arabinose, L-citrulline, L-methionine, L-rhamnose, L-serine, L-sorbose, L-threonine, L-tryptophan, maltose, melezitose, melibiose, myo-inositol, nicotinic acid, p-arbutin, protocatechuic acid, raffinose, ribitol, sorbitol, sucrose, thymine, uridine, vanillin, xylitol) as the sole carbon source.

P. resinovorans (genotype II) SZMC 25875 grew well in the presence of 13 compounds (2-keto-D-gluconic acid, D-glucose, DL-isocitric acid, gluconic acid, L-arginine, L-asparagine, L-glutamic acid, L-glutamine, L-lactic acid (Na-DL-lactate), L-malic acid, L-proline, sodium pyruvate, succinic acid) with OD_{620} values over 0.2, while in the case of 7 compounds (beta-alanine, glycerol-l-monoacetate, ketoisovaleric acid, L-lysine, L-valine, starch, tannic acid) moderate (OD_{620} = 0.1-0.2), while in the case of 9 substances (cytidine, D-galactose, glycine, L-citrulline, L-histidine, L-ornithine, thymine, xylan, xylitol) weak growth was shown (OD_{620} between 0.05-0.1). No growth was detected in the presence of 51 compounds ((-) quinic acid, adenosine, alpha-methyl-D-mannoside, ascorbic acid, beta-methyl-D-galactoside, cellobiose, cis-aconitic acid, cytosine, D-arabinose, dextran, D-fructose, D-glucuronic acid, D-glucosamine, dihydroxyacetone, D-lyxose, D-mannitol, D-mannose, D-xylose, ethanol, fumaric acid, galactitol, gallic acid, gamma-butyrolactone, gentisic acid, glycerol, i-erythritol, inosine, inulin, lactose, L-alanine, L-arabinose, L-isoleucine, L-methionine, L-rhamnose, L-serine, L-sorbose, L-threonine, L-tryptophan,

maltose, melezitose, melibiose, myo-inositol, nicotinic acid, p-arbutin, protocatechuic acid, raffinose, ribitol, sorbitol, sucrose, uridine, vanillin) as carbon source.

A. tumefaciens SZMC 14557 utilised 18 compounds (2-keto-D-gluconic acid, D-fructose, D-mannitol, glycerol, glycerol-1-monoacetate, lactose, L-asparagine, L-glutamine, L-malic acid, maltose, melezitose, melibiose, myo-inositol, p-arbutin, raffinose, ribitol, succinic acid, sucrose) with OD_{620} values above 0.2, 28 compounds (beta-methyl-D-galactoside, cellobiose, D-arabinose, D-galactose, D-glucuronic acid, D-glucosamine, D-glucose, D-lyxose, D-mannose, D-xylose, ethanol, galactitol, L-alanine, L-arabinose, L-glutamic acid, L-lactic acid, L-proline, L-rhamnose, L-serine, sodium pyruvate, sorbitol, starch, tannic acid, uridine, xylitol) with OD_{620} in the range 0.1-0.2, and 6 compounds (dextran, ketoisovaleric acid, L-histidine, L-sorbose, vanillin, xylan) with low OD_{620} values (0.05-0.1). The following 31 compounds were not utilised as carbon source ((-) quinic acid, adenosine, alpha-methyl-D-mannoside, ascorbic acid, beta-alanine, cis-aconitic acid, cytidine, cytosine, dihydroxyacetone, DL-isocitric acid, fumaric acid, gallic acid, gamma-butyrolactone, gentisic acid, gluconic acid, glycine, i-erythritol, inosine, inulin, L-arginine, L-citrulline, L-isoleucine, L-lysine, L-methionine, L-ornithine, L-threonine, L-tryptophan, L-valine, nicotinic acid, protocatechuic acid, thymine).

A. vitis SZMC 21396 grew in the presence of only 3 compounds (maltose, succinic acid, tannic acid) at the highest rate (OD_{620} >0.2), 20 substances (D-fructose, D-galactose, D-glucose, D-lyxose, D-mannitol, D-mannose, D-xylose, glycerol-l-monoacetate, L-arabinose, L-glutamic acid, L-glutamine, L-malic acid, L-rhamnose, melibiose, p-arbutin, raffinose, ribitol, sorbitol, starch, sucrose) at moderate rate (OD_{620} 0.1-0.2), and 6 compounds (cellobiose, glycerol, L-alanine, L-proline, myo-inositol, sodium pyruvate) at the low rate (OD_{620} 0.05-0.1). The strain did not utilise 51 compounds ((-) quinic acid, 2-keto-D-gluconic acid, adenosine, alpha-methyl-D-mannoside, ascorbic acid, beta-alanine, beta-methyl-D-galactoside, cis-aconitic acid, cytidine, cytosine, D-arabinose, dextran, D-glucuronic acid, D-glucosamine, dihydroxyacetone, DL-isocitric acid, ethanol, fumaric acid, galactitol, gallic acid, gamma-butyrolactone, gentisic acid, gluconic acid, glycine, i-erythritol, inosine, inulin, ketoisovaleric acid, lactose, L-arginine, L-asparagine, L-citrulline, L-histidine, L-isoleucine, L-lactic acid, L-lysine, L-methionine, L-ornithine, L-serine, L-sorbose, L-threonine, L-tryptophan, L-valine, melezitose, nicotinic acid, protocatechuic acid, thymine, uridine, vanillin, xylan, xylitol).

In the current literature, data are not available about the carbon source utilisation profile of either *P. resinovorans* or *E. adhaerens*. Therefore, the obtained findings might be compared to other reports only indirectly, by analysing the composition of media which were used to grow these species in the corresponding references. *E. adhaerens* OS3 was capable of hydrolysing starch, and utilizing carbohydrates such as glucose, sucrose, and mannitol (Oves et al. 2017). Furthermore, this species was reported to be promising for the biodegradation of the insecticides thiamethoxam and flonicamid (Zhou et al. 2013, Zhao et al. 2021) and polychlorinated biphenyls (Xu et al. 2016), as it could utilise these compounds as carbon sources.

P. resinovorans SPR1 was able to utilise eugenol as a source of energy by transforming it into vanillin, which broadens its biotechnological potential (Ashengroph et al. 2011). Another strain of *P. resinovorans*, CA10 was mentioned in the literature to be able to degrade carbazole by the utilisation of this compound as carbon and nitrogen source (Shintani et al. 2013). *P. aeruginosa* GS-33 was found to utilise glucose, xylose, fructose, galactose, melibiose, mannose, glycerol, mannitol, xylitol, and malonate, but maltose, raffinose, trehalose, sucrose, inulin, sodium gluconate, salicin, dulcitol, inositol, erythritol, α-methyl-D-glucoside, rhamnose, cellobiose, melezitose, α-methyl D-mannoside, ONPG, D-arabinose and sorbose could not be utilised by the strain as carbon source (Patil et al. 2016). Among the 80 tested carbon sources, all strains were able to utilise 10 compounds, namely D-glucose, L-

alanine, L-glutamic acid, L-glutamine, L-malic acid, L-proline, sodium pyruvate, starch, succinic acid and tannic acid at a certain level (OD_{620} values varied between 0.050 and >0.2, depending on the strain), while none of them was able to utilise 11 compounds ((-) quinic acid, ascorbic acid, cis-aconitic acid, cytosine, fumaric acid, gallic acid, gentisic acid, inosine, L-methionine, nicotinic acid, and protocatechuic acid) as the sole carbon source. Thirteen compounds, viz. 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 (either SZMC 25872 or SZMC 25875, the most promising biocontrol agents, and *A. tumefaciens* SZMC 14557 (the most susceptible pathogenic strain, Subsection 6.4.2) were chosen to test the effect of carbon sources on the inhibition of *A. tumefaciens* by *P. resinovorans* (Subsection 6.4.4).

6.4.4 The influence of carbon sources on the antagonistic potential of glyphosate-tolerant bacteria against *A. tumefaciens*

The influence of different carbon sources on the suppression of *A. tumefaciens* SZMC 14557 by *P. resinovorans* (genotype I) SZMC 25872 and *P. resinovorans* (genotype II) SZMC 25875were evaluated using 13 compounds, which were utilised as sole carbon source by both the pathogenic and the glyphosate-tolerant strains in liquid cultures (Subsection 6.4.3). On solid media, *P. resinovorans* SZMC 25872 could grow in the presence of most of the tested compounds, except for glycerol-l-monoacetate, L-malic acid, and starch (Table 14). The growth of *A. tumefaciens* SZMC 14557 was substantially inhibited by *P. resinovorans* SZMC 25872 on all utilised compounds, with the highest inhibition zones of 17.67, 20.00, 25.33, and 19.00 mm on plates containing D-glucose, L-alanine, sodium pyruvate, and succinic acid, respectively (Table 14). *P. resinovorans* SZMC 25875 grew only in the presence of 2-keto-D-gluconic acid, D-glucose, L-asparagine, L-glutamic acid, L-glutamine, sodium pyruvate, and succinic acid. This strain showed no antagonistic activity towards *A. tumefaciens* SZMC 14557 on any plates, except for the positive control (PDA, Table 14). As only partial inhibition was observed in the case of *A. vitis*, the effect of carbon sources on the biocontrol efficacy of *P. resinovorans* against this species was not evaluated.

Table 14. Colony diameter (C, mm) and colony+inhibition zone diameter (IZ, mm) caused by *P. resinovorans* SZMC 25872 and SZMC 22875 on *A. tumefaciens* SZMC 14557 in the presence of different carbon sources (means of 3 replicates±SD)

Carbon source	***P. resinovorans* isolates**			
	SZMC 25872 (genotype I)		**SZMC 25875 (genotype II)**	
	C	**IZ**	**C**	**IZ**
PDA	9.00±0.00	20.67±1.15	8.33±0.58	19.67±2.31
2-keto-D-gluconic acid	7.67±0.58	15.33±1.53	7.33±0.58	0.00±0.00
D-fructose	8.33±0.58	9.00±7.81	0.00±0.00	0.00±0.00
D-glucuronic acid	9.33±0.58	23.67±1.53	0.00±0.00	0.00±0.00
D-glucose	8.67±0.58	17.67±0.58	9.33±0.58	0.00±0.00
Glycerol-l-monoacetate	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
L-alanine	9.33±0.58	20.00±4.58	0.00±0.00	0.00±0.00
L-asparagine	9.33±0.58	17.00±1.73	8.67±0.58	0.00±0.00
L-glutamic acid	9.67±0.58	17.33±3.79	9.33±0.58	0.00±0.00
L-glutamine	9.67±0.58	16.33±3.51	9.00±0.00	0.00±0.00
L-malic acid	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Sodium pyruvate	10.33±0.58	25.33±1.53	11.00±0.00	0.00±0.00
Starch	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Succinic acid	9.00±0.00	19.00±1.00	9.33±0.58	0.00±0.00

The composition of the growth medium, especially carbon source plays an important role in the biocontrol activity of microorganisms, particularly on the production of antibiotics. Glycerol as a sole carbon source suppressed the expression of the gene encoding for the 7-hydroxytropolone (pyoverdine-type siderophore), which is involved in the antimicrobial activity of *P. donghuensis* P482 (Matuszewska et al. 2021). In contrast, glycerol and glucose promoted the synthesis of the antibiotics 2,4-diacetylphloroglucinol, pyoluteorin, and pyoluteorin by *P. fluorescens* CHA0 (Duffy and Défago 1999). The effect of carbon source might depend on the species of the potential biocontrol strain. The siderophores and antibiotics produced by *P. aeruginosa* D4 and *B. stratosphericus* FW3 inhibited the growth of a variety of plant pathogens, including *Burkholderia glumae* KACC 10 138, *X. oxyzae* pv. *oryzae* KACC 10 208, *P. syringae* KACC 15 105, *Pectobacterium carotovorum* KACC 17 004 and *Ralstonia solanacearum* KACC 10 718. Among the tested carbon sources (lactose, sucrose, starch, and glucose), the inhibition pattern of *P. aeruginosa* D4 was almost the same in the presence of lactose, sucrose, starch, and glucose, however, no inhibition of *R. solanacearum* was detected in the case of starch and glucose. In contrast, in the case of *B. stratosphericus*, the highest inhibition of *R. solanacearum* was found with starch, while *P. carotovorum* was not suppresed in the presence of lactose, and the inhibition of *B. gluma* and *P. syringae* was similar with all tested carbon sources (Durairaj et al. 2017).

Our studies have confirmed the importance of carbon sources in the biocontrol efficacy. Among the 13 tested carbon-containing compounds, the inhibition zone caused by *P. resinovorans* SZMC 25872 in the colony of *A. tumefaciens* fell in the range of 9.0 (D-fructose) - 25.33 mm (sodium pyruvate). Subsequently, the culture supernatants obtained from MM amended with the carbon sources resulting in the highest degree of inhibition (namely, sodium pyruvate, L-alanine, succinic acid, and D-glucose) as well as the other media applied in the biocontrol studies media (PDB, LB, and YEG) were selected for studying the biocontrol activity, to examine the mode of inhibition mode and to identify the compounds responsible for the inhibitory effect (Subsections 6.4.5-6.4.9).

6.4.5 Testing the inhibitory effect of the CCF of *P. resinovorans* strains on the growth of *A. tumefaciens*

Among the 7 different tested conditions, L-alanine and succinic acid as sole carbon sources were found to enhance the growth inhibition of *A. tumefaciens* SZMC 14557 by the selected *P. resinovorans* strains. Interestingly, a significant decrease in the growth of *A. tumefaciens* (15-30%) was also observed in comparison with the positive control in the presence of 25-50% CCF of non-inoculated control samples obtained from these media ($p<0.05$) (Table 15). However, the inhibition rate caused by 25-50% CCF of *P. resinovorans* strains was significantly different from both values of the positive control and the inhibition due to the negative control ($p<0.05$). Comparing to the positive control, CCF samples obtained from MM amended with L-alanine of both *P. resinovorans* strains at 25% concentration resulted in more than 50% significant growth inhibition of the pathogenic strain ($p<0.05$), while increasing the concentration of CCFs to 50% led to almost complete suppression of *A. tumefaciens* SZMC 14557 ($p<0.05$) (Table 15). Furthermore, 50% succinic acid-containing CCF samples of both SZMC 25872 and SZMC 25875 also significantly inhibited the growth of *A. tumefaciens*, comparing to the positive control ($p<0.05$). The PDB-derived CCF of *P. resinovorans* SZMC 25872 also showed significant inhibition at 12.5-50% ($p<0.05$). No growth suppression was observed by the CCF samples of the examined glyphosate-tolerant strains obtained from YEG, LB, and glucose even at the highest tested concentration (Table 15).

Table 15. Cell density (OD_{620}) and growth inhibition (%) of *A. tumefaciens* SZMC 14557 in the presence of 12.5-50% CCF of 3 glyphosate-tolerant bacteria (mean of 3 replicates±SD)

CCF, %	Non-inoculated control		*E. adhaerens* SZMC 25856		*P. resinovorans* (genotype I) SZMC 25872		*P. resinovorans* (genotype II) SZMC 25875	
	OD_{620}	Inhibition, %	OD_{620}	Inhibition, %	OD_{620}	Inhibition, %	OD_{620}	Inhibition, %
PDB								
50	0.523±0.06	0.57±11.10	0.538±0.04	12.75±6.42	0.501±0.04	24.78±6.69	0.541±0.02	9.93±4.07
25	0.528±0.02	-0.38±3.67	0.516±0.03	16.32±4.65	0.544±0.06	18.37±8.79	0.543±0.04	9.65±6.81
12.5	0.570±0.02	-8.43±4.67	0.538±0.01	12.75±1.75	0.550±0.00	17.42±0.65	0.524±0.03	12.76±4.19
0	0.526±0.03	-	0.617±0.04	-	0.666±0.01	-	0.601±0.06	-
LB								
50	0.626±0.05	-1.68±7.79	0.518±0.02	12.35±3.01	0.671±0.03	-20.47±5.15	0.595±0.05	-2.53±7.89
25	0.575±0.03	6.61±5.23	0.579±0.01	1.97±2.48	0.587±0.01	-5.33±1.67	0.585±0.04	-0.86±6.92
12.5	0.547±0.01	11.15±1.38	0.548±0.00	7.28±0.45	0.575±0.01	-3.23±1.08	0.522±0.04	9.94±6.39
0	0.616±0.05	-	0.591±0.02	-	0.557±0.01	-	0.580±0.04	-
Glucose								
50	0.504±0.02	13.61±3.70	0.497±0.02	16.75±3.86	0.541±0.04	15.15±6.64	0.567±0.01	11.90±1.40
25	0.519±0.04	10.92±6.97	0.555±0.02	6.98±3.67	0.573±0.02	10.24±3.00	0.576±0.03	10.61±4.79
12.5	0.552±0.02	5.32±3.82	0.570±0.00	4.52±0.34	0.592±0.02	7.21±3.15	0.567±0.06	11.90±9.47
0	0.583±0.02	-	0.597±0.04	-	0.638±0.02	-	0.644±0.04	-
L-alanine								
50	0.396±0.02	33.78±2.75	0.353±0.02	37.36±3.01	0.094±0.00	83.03±0.37	0.085±0.00	85.63±0.68
25	0.435±0.01	27.20±2.18	0.397±0.01	29.43±2.49	0.230±0.02	58.69±3.80	0.160±0.04	73.01±6.95
12.5	0.494±0.01	17.34±1.68	0.455±0.02	19.12±3.02	0.337±0.02	39.45±4.41	0.316±0.04	46.80±5.95
0	0.598±0.04	-	0.563±0.02	-	0.556±0.01	-	0.594±0.01	-
Sodium pyruvate								
50	0.420±0.04	25.53±7.26	0.507±0.01	19.60±1.62	0.450±0.06	30.56±9.99	0.555±0.02	12.37±3.59
25	0.518±0.02	8.22±3.38	0.591±0.02	6.39±3.78	0.633±0.02	2.26±3.77	0.588±0.01	7.06±1.19
12.5	0.524±0.04	7.03±7.12	0.615±0.00	2.48±0.48	0.593±0.01	8.49±1.93	0.567±0.02	10.43±3.56
0	0.564±0.06	-	0.631±0.04	-	0.648±0.05	-	0.633±0.01	-
Succinic acid								
50	0.461±0.01	23.68±1.44	0.458±0.02	18.15±4.44	0.244±0.01	56.80±1.49	0.466±0.03	21.42±5.44
25	0.508±0.01	15.89±1.41	0.524±0.01	6.37±0.98	0.435±0.02	22.81±3.99	0.533±0.01	10.06±2.46
12.5	0.584±0.07	3.31±11.42	0.528±0.02	5.77±3.82	0.547±0.04	3.07±7.93	0.495±0.02	16.58±3.47
0	0.604±0.04	-	0.560±0.03	-	0.564±0.01	-	0.593±0.01	-
YEG								
50	0.513±0.05	-0.39±8.92	0.477±0.04	6.84±8.63	0.566±0.01	-13.82±1.93	0.528±0.02	-5.46±3.77
25	0.517±0.04	-1.11±7.68	0.522±0.00	-1.89±0.49	0.565±0.02	-13.68±4.55	0.523±0.01	-4.39±1.56
12.5	0.529±0.03	-3.52±5.93	0.541±0.02	-5.60±4.30	0.529±0.01	-6.44±1.39	0.521±0.02	-3.99±4.21
0	0.511±0.01	-	0.512±0.02	-	0.497±0.01	-	0.501±0.00	-

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 the metabolites produced by *P. resinovorans* with antimicrobial activity against *A. tumefaciens*. *P. resinovorans* SZMC 25872 was proposed as the most potential biocontrol strain, since its CCF samples obtained from cultivation in the presence of L-alanine and succinic acid as the sole carbon sources could significantly inhibit the growth of *A. tumefaciens* SZMC 14557 ($p<0.05$) at 25-50% and 50% concentrations, respectively. Therefore, this strain was selected for further eco-physiological characterisation together with isolates *E. adhaerens* SZMC 25856 and *P. resinovorans* SZMC 25875, which showed plant growth-promoting potential in the previous experiments (Section 6.3). Moreover, all tested CCFs (Table 15) were subjected to HPLC-HRMS analysis (Subsection 6.4.9) as well as for the semi-quantitative determination of siderophore production (Subsection 6.4.6) and extracellular enzyme activity tests (Subsection 6.4.8). Being the filtrates showing the highest inhibitory effect on *A. tumefaciens*, the CCF samples obtained from cultivation in MM amended with L-alanine and succinic acid of both *P. resinovorans* strains were selected for testing the hypothesis about siderophore and ROS-mediated antagonistic activity as well as to test the effect of heat treatment on the inhibitory activity of detected metabolites compounds (Subsection 6.4.7).

The culture supernatants, different extracts, and filtrates of certain bacteria were previously reported to have inhibitory effect on phytopathogenic microorganisms. The benzene fraction obtained from the culture supernatant of *P. chlororaphis* ssp. *aureofaciens* DSM 6698 completely inhibited the growth of *R. solani*, *P. ultimum*, and *F. oxysporum* (Mezaache-Aichour et al. 2013). Different antimicrobial compounds, such as phenazines and siderophores were found to play role in the antagonistic effect of eleven isolates of fluorescent pseudomonads, including *P. fluorescens*, *P. aeruginosa*, *P. chlororaphis* ssp. *aurantiaca*, and *P. chlororaphis* ssp. *chlororaphis* (Shahid et al. 2021). Lee et al. (2016) reported about the inhibitory effect of the culture filtrates of a *P. aeruginosa* strain on the growth of *Corynebacterium glutamicum*, *B. subtilis*, *S. aureus*, and *A. tumefaciens*..

Therefore, as CCFs of *P. resinovorans* SZMC 25872 and SZMC 25875 obtained from mininal medium amended individually with L-alanine and succinic acid showed significant inhibition of *A. tumefaciens* SZMC 14557, these filtrates are proposed to contain inhibitory compounds. Furthermore, as *P. resinovorans* is presented by us as a species with biocontrol potential for the first time, its CCFs are expected to contain novel, yet unidentified bioactive metabolites which are to be examined by HPLC-MS analysis (Subsection 6.4.9).

6.4.6 Siderophore production by *P. resinovorans* strains cultivated in different media correlated with the growth inhibition of *A. tumefaciens*

The siderophore content of the CCF samples showing inhibitory effect on the growth of *A. tumefaciens* SZMC 14557 (Subsection 6.4.5) were analysed by the CAS colourimetric method. The degree of inhibition caused by 25 and 50% CCFs are presented in Table 16 together with the data of siderophore production.

Table 16. Detection of siderophores produced by *E. adhaerens* SZMC 25856, *P. resinovorans* (genotype I) SZMC 25872, and *P. resinovorans* (genotype II) SZMC 25875 in CCF samples, and the growth inhibition (%) of *A. tumefaciens* SZMC 14557 caused by 25 and 50% concentrations of CCF (means of 3 replicates±SD)

Media	Strains	Siderophore production, %	Inhibition rate (%) due to CCFs applied at different concentrations	
			25%	50%
PDB	SZMC 25856	-65.39±37.24	16.32±4.65	12.75±6.42
	SZMC 25872	-43.73±11.36	18.37±8.79	24.77±6.69
	SZMC 25875	-52.74±2.40	9.65±6.81	9.93±4.07
LB	SZMC 25856	-59.14±11.76	1.97±2.48	12.35±3.01
	SZMC 25872	-2.01±3.72	-5.33±1.67	-20.47±5.15
	SZMC 25875	-67.00±2.63	-0.86±6.92	-2.53±7.89
MM+glucose	SZMC 25856	-13.60±9.03	6.98±3.67	16.75±3.86
	SZMC 25872	-15.63±6.10	10.24±3.00	15.15±6.64
	SZMC 25875	-15.53±9.39	10.61±4.79	11.90±1.40
MM+L-alanine	SZMC 25856	7.54±3.40	29.43±2.49	37.36±3.01
	SZMC 25872	86.30±1.69	58.69±3.80	83.03±0.37
	SZMC 25875	33.57±10.79	73.01±6.95	85.63±0.68
MM+sodium pyruvate	SZMC 25856	47.13±3.07	6.39±3.78	19.60±1.62
	SZMC 25872	78.97±5.54	2.26±3.77	30.56±9.99
	SZMC 25875	66.67±2.15	7.06±1.19	12.37±3.59
MM+succinic acid	SZMC 25856	34.50±5.02	6.37±0.98	18.15±4.44
	SZMC 25872	80.41±6.39	22.81±3.99	56.80±1.49
	SZMC 25875	39.13±1.75	10.06±2.46	21.42±5.44
YEG	SZMC 25856	-77.78±4.22	-1.89±0.49	6.84±8.63
	SZMC 25872	-64.81±11.01	-13.68±4.55	-13.82±1.93
	SZMC 25875	-84.53±4.19	-4.39±1.56	-5.46±3.77

The data of growth inhibition are presented as the decrease in the OD_{620} values of the treated samples in comparison with the positive control (PDB without CCF) calculated by the following formula: (Positive control − treated sample) × 100 ⁄ positive control.

The remarkable influence of the different growth media and carbon sources on siderophore production was described in Subsection 6.3.3. The highest siderophore amount was detected in the presence of succinic acid followed by L-alanine and sodium pyruvate. Importantly, the data presented in Table 16 clearly shows considerable inhibition of *A. tumefaciens* by samples with remarkable siderophore production. The highest degree of significant growth inhibition ($p<0.05$) due to CCFs with L-alanine and succinic acid could be associated with the siderophores detected in these samples. In contrast, no siderophores were found in PDB, LB, YEG, and MM-glucose, and consequently no significant inhibition of *A. tumefaciens* SZMC 14557 ($p>0.05$) was observed following treatment with the CCFs obtained from these media, except for the CCF of *P. resinovorans* (genotype I) SZMC 25872 from PDB. The siderophore amount and growth inhibition due to 25 and 50% concentrations of CCF showed significant correlation ($r = 0.32$, $p<0.05$ and $r = 0.54$, $p<0.001$ for 25 and 50% CCF, respectively) in the statistical analysis (Kendall's rank correlation tau) (Figure 9).

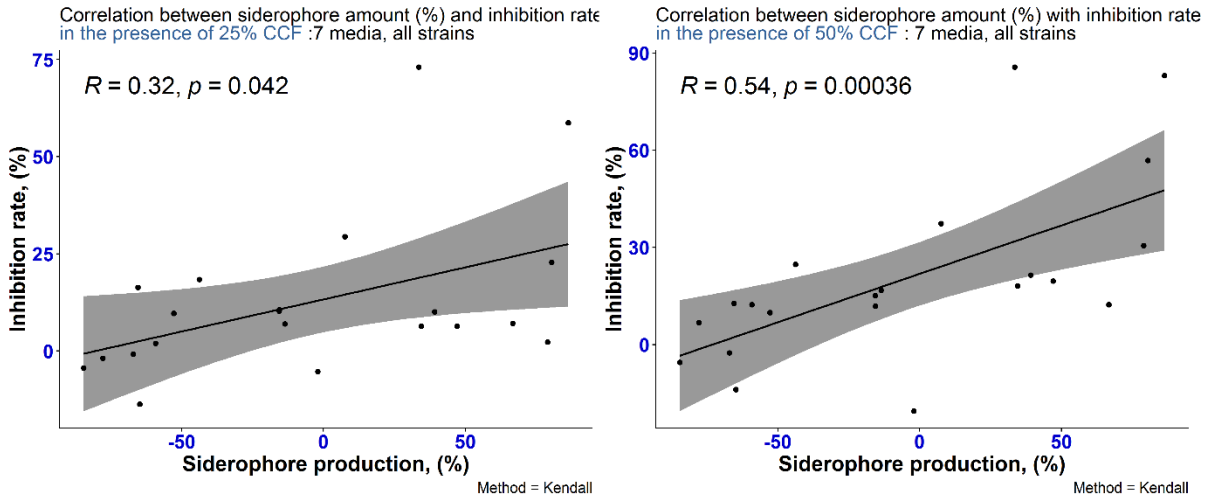


Figure 9. Analysis of correlation between the siderophore production of glyphosate-tolerant strains and the growth inhibition of *A. tumefaciens* SZMC 14557 by 25% (left) and 50% (right) CCF (Table 16), Kendall coefficient. Grey colour represents 95% confidence intervals.

Although, considerable amount of siderophores was detected also in MM+sodium pyruvate, no remarkable inhibition was found in these CCF samples. It might be explained by the lower OD_{630} value of the reference sample: non-inoculated supernatant itself reacted well with CAS solution, and this reaction resulted in lower OD_{630}, and consequently in higher values of siderophore percentage in the inoculated samples. However, insignificant inhibition ($p>0.05$) up to 30% was still observed in the samples treated with CCF from MM+sodium pyruvate. Remarkable siderophore production was observed in CCFs of *P. resinovorans* SZMC 25872 and SZMC 25875 grown in MM amended with L-alanine (86.30-33.57%) and succinic acid (80.41-39.13%), respectively. Significant correlation ($r = 0.32$, $p<0.05$; and $r = 0.54$, $p<0.001$) was found between these values and the growth inhibition of *A. tumefaciens* due to treatment with CCFs at 25 and 50% concentrations, respectively. Therefore, as siderophores were reported to be involved in the suppression of numerous pathogenic species (Kloepper et al. 1980, Lee et al. 2016, Tao et al. 2020) including *A. tumefaciens* (Penyalver et al. 2001) (Subsection 3.2.4), and as the enantio-pyochelin siderophore produced by *P. protegens* CS1 showed inhibitory effect against *X. citri* ssp. *citri* via a ROS-mediated pathway (Michavila et al. 2017), siderophore-mediated suppression may be suggested as a potential mechanism involved in the growth inhibition of *A. tumefaciens* SZMC 14557. Therefore, the follow-up studies were aimed at the determination of the potential role of siderophores produced by *P. resinovorans* in the suppression of *A. tumefaciens* SZMC 14557 (Subsection 6.4.7). The amendment of the culture media with additional iron and ascorbic acid were proposed to eliminate the action of siderophores as iron-removing or ROS-generating agents, respectively, in order to test the possibility of siderophore-mediated suppression of *A. tumefaciens* SZMC 14557 by *P. resinovorans* strains. Heat treatment (80 and 90 °C) was applied to check thermal stability of the potential active metabolites.

6.4.7 Testing the hypothesis of the siderophore- and ROS-mediated suppression of the growth of *A. tumefaciens* and the inhibitory effect of CCF after heat-treatment

In this assay, inhibition pattern was the same as described for testing the inhibitory effect of 12.5-50% CCF on the growth of *A. tumefaciens* SZMC 14557 (Subsection 6.4.5). Addition of 25 and 50% of non-inoculated CCF obtained from L-alanine resulted in significant inhibition of *A. tumefaciens* ($p<0.05$) compared to the positive control (Figure 10).

However, the presence of CCF from the same culture media of *P. resinovorans* SZMC 25872 (genotype I) and SZMC 25875 (genotype II) at either 25 or 50% also resulted in significant growth suppression compared to both the positive control and the non-inoculated CCF ($p<0.05$). Moreover, 50% CCF of *P. resinovorans* (genotype I) SZMC 25872 obtained from MM amended with succinic acid also caused significant inhibition ($p<0.05$).

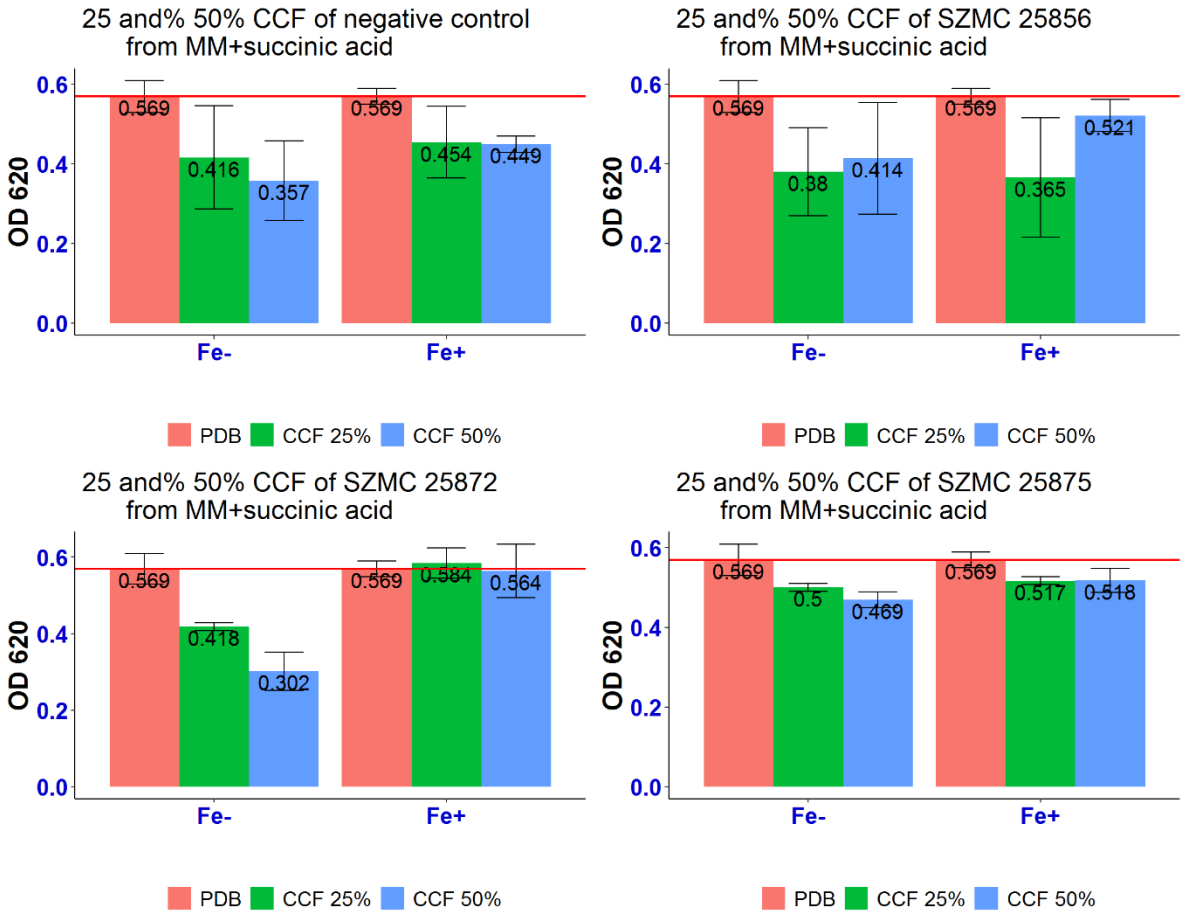


Figure 10. The effect of 25 and 50% CCF of the control (non-inoculated medium) and 3 glyphosate-tolerant bacteria obtained from MM amended with succinic acid on the growth of *A. tumefaciens* SZMC 14557 in the absence (Fe-) and presence (Fe+) of an extra iron source (29 mg/l $FeCl_3 \cdot 6H_2O$). PDB is positive control (no added CCF), CCF 25% and CCF 50% are the corresponding CCF concentrations. The red line represents the lowest value of the positive control.

Interestingly, the addition of 29 mg/l $FeCl_3 \cdot 6H_2O$ to samples treated with 25 and 50% CCF of *P. resinovorans* SZMC 25872 obtained from succinic acid-containing medium caused the significantly increased growth of *A. tumefaciens* compared to the growth of same sample without extra iron source ($p<0.05$). The growth of *A. tumefaciens* SZMC 14557 treated with 50% CCF of SZMC 25872 from MM-succinic acid was significantly inhibited ($p<0.05$) compared to positive control, the growth of pathogen treated with same sample in the presence of an additional iron source was significantly higher than sample without amendment with iron ($p<0.05$), consequently, it was not significantly different from positive control ($p>0.05$). In addition, an extra iron source did not promote the growth of the positive control itself: no significant difference was found between the growth in positive control (pure PDB containing no CCF) samples with and without the addition of $FeCl_3 \cdot 6H_2O$ ($p>0.05$). Therefore, significant stimulatory effect ($p<0.05$) of the extra iron on the bacterial growth was observed only when *A. tumefaciens* was treated with 25 and 50% CCF of *P.*

resinovorans SZMC 25872 containing the highest siderophore amount (Table 16, Subsection 6.4.6). Altogether, these results supported our hypothesis about siderophore-mediated suppression of *A. tumefaciens* by *P. resinovorans* SZMC 25872, because inhibitory effect caused by CCF was eliminated by the presence of extra iron.

Siderophore production is a well-known component of the suppression of plant pathogens by microbial biocontrol agents. Limiting the availability of iron leads to the restriction of bacterial growth (O'Sullivan and O'Gara 1992; Saha et al. 2016), the details of this mechanism are described in Subsection 3.2.4. Importantly, among the 3 types of siderophores, namely hydroxamates, catecholates, and carboxylates (Saha et al. 2016), the pathogenic and beneficial strains have to utilise different types, otherwise the siderophores produced by the biocontrol strain might be exploited by the pathogen, rather than being suppressed due to their action. *A. tumefaciens* was reported to be a catecholate siderophore-producing species with its specific siderophore agrobactin (Leong and Neilands 1981, 1982; Sonoda et al. 2002). The siderophores produced by both *E. adhaerens* TMX-23 and OS3 strains (salicylic acid and dihydrobenzoic acid) were classified to the group of catechols (Zhou et al. 2013, Oves et al. 2017). Based on these findings, it can be assumed that *E. adhaerens* SZMC 25856 might also produce catechol-type siderophores, which would explain the lack of the inhibitory effect of this strain on *A. tumefaciens*. Changes in colour of halo zone on CAS agar plates from blue to purple and orange were attributed to the action of catechol or hydroxamate siderophores, respectively (Pérez-Miranda et al. 2007). Therefore, as all the investigated *P. resinovorans* strains produced orange halozones in the CAS agar assays (Figure 7, Subsection 6.3.3) they are likely to have produced hydroxamate-type siderophores, which cannot be exploited by *A. tumefaciens* SZMC 14557, resulting in its limited growth. The hydroxamate-type siderophore agrocin 434, produced by *A. radiobacter* K84, inhibited pathogenic *A. tumefacine*s strains (Penyalver et al. 2001). It is obvious that the amount of siderophores is also a crucial factor in the siderophore-mediated suppression of pathogens. In our findings, the highest siderophore amount was detected in the CCF samples of the *P. resinovorans* strains obtained from MM amended with succinic acid and L-alanine as the sole carbon source. Siderophores produced under these conditions are likely to have played a role in the inhibition of *A. tumefaciens* SZMC 14557. Similarly to our results, the siderophore amount produced by *P. aeruginosa* KA19 was the highest in medium amended with succinate among 6 different carbon sources, including glucose, fructose, and mannitol. This strain significantly reduced black rot lesions caused by *X. campestris* pv. *campestris* compared to the untreated control (Mishra and Arora 2012).

However, no significant increase in the growth of *A. tumefaciens* SZMC 14557 was observed between samples treated with 25-50% CCF of all strains obtained from MM containing L-alanine regardless of the presence or absence of an extra iron source (Figure 11).

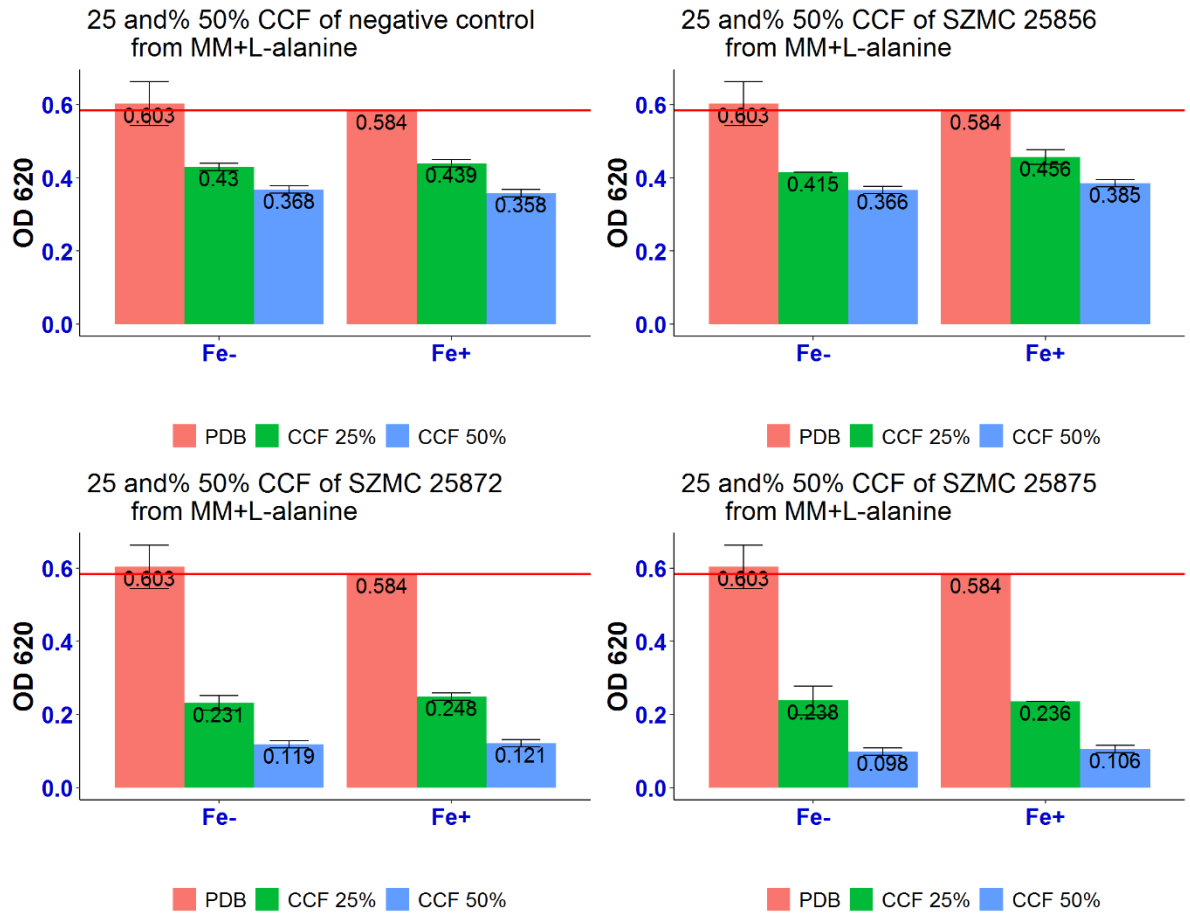


Figure 11. The effect of 25 and 50% CCF of the control (non-inoculated medium) and 3 glyphosate-tolerant bacteria obtained from MM amended with L-alanine on the growth of *A. tumefaciens* SZMC 14557 in the absence (Fe-) and presence (Fe+) of an extra iron source (29 mg/l $FeCl_3 \cdot 6H_2O$). PDB is positive control (no added CCF), CCF 25% and CCF 50% are the corresponding CCF concentrations. The red line represents the lowest value of the positive control.

In comparison with the positive control, CCF of non-inoculated sample and *P. resinovorans* SZMC 25856 significantly inhibited SZMC 14557 ($p<0.05$) at both 25 and 50% concentrations, while those of SZMC 25872 and SZMC 25875 applied at 25 and 50% resulted in a significant growth suppression of *A. tumefaciens* compared to both the positive control, as well as the inhibition caused by the negative control and *P. resinovorans* SZMC 25856 ($p<0.05$). The same inhibition pattern was found in all samples regardless of the presence of an additional iron source. These findings suggest different modes of inhibition caused by CCFs obtained from the culture supernatants of *P. resinovorans* grown in the presence of succinic acid and L-alanine. The inhibitory effect of CCF from MM+succinic acid could be eliminated by the addition of 29 mg/l extra iron, supporting the siderophore-mediated antagonism, reported in several previous studies (Rane et al. 2008, Mishra and Arora 2012, Sulochana et al. 2014, Michavila et al. 2017). The growth suppression by CCF from MM+L-alanine remained the same in the presence of an additional iron, which may be the consequence of further inhibitory compound(s) produced by *P. resinovorans* grown in the presence of L-alanine as the sole carbon source.

The siderophores pyochelin and enantio-pyochelin produced by *P. aeruginosa* PAO1 and *P. protegens* CS1, respectively, were proposed to cause ROS generation leading to lipid peroxidation and the suppression of the plant pathogenic *X. citri* subsp. *citri* via a ROS-mediated pathway (Adler et al. 2012, Michavila et al. 2017). Enantio-pyochelin resulted in a

significantly higher degree of ROS generation and lipid peroxidation in *X. citri* subsp. *citri*, but the addition of 1 mM ascorbic acid as a ROS-scavenging agent reduced the level of both parameters, which led to the elimination of growth suppression (Michavila et al. 2017). Based on these findings, the follow-up studies were performed to test the potential role of CCF in the ROS-mediated growth suppression of *A. tumefaciens*. Samples were treated with ascorbic acid to scavenge ROS possibly generated due to the presence of CCF, and thus to prevent *A. tumefaciens* from this oxidative stress factor.

The addition of ascorbic acid did not promote the growth of the positive control ($p>0.05$). Furthermore, ascorbic acid did not affect significantly the growth of the majority of the samples ($p>0.05$ between the bacterial growth in samples with and without vitamin C), except for the significant positive influence ($p<0.05$) of CCF (25 and 50%) on the negative control, *E. adhaerens* SZMC 25856 at 50% CCF, and *P. resinovorans* (genotype II) SZMC 25875 at 25% CCF (in all cases, CCF were obtained from MM+succinic acid, Figure 12). However, this increase was not sufficient to eliminate the inhibitory effect of CCF from both MM+succinic acid and L-alanine applied at 25 and 50% concentrations.

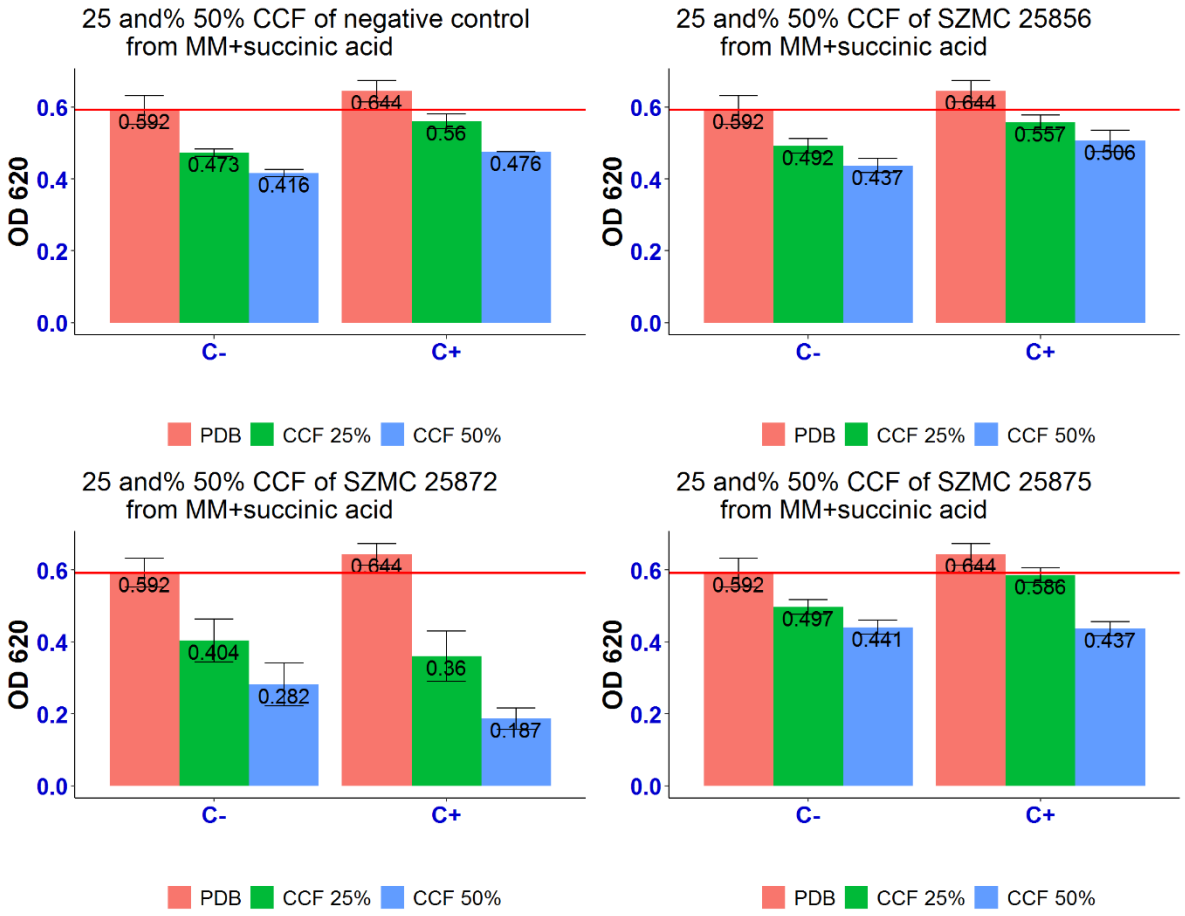


Figure 12. The effect of 25 and 50% CCF of the control (non-inoculated medium) and 3 glyphosate-tolerant bacteria obtained from MM amended with succinic acid on the growth of *A. tumefaciens* SZMC 14557 in the absence (C-) and presence (C+) of 1 mM ascorbic acid. PDB is positive control (no added CCF), CCF 25% and CCF 50% are the corresponding CCF concentrations. The red line represents the lowest value of the positive control.

In the case of MM+succinic acid, the presence of 25 and 50% CCF from the non-inoculated sample, as well as *P. resinovorans* SZMC 25856 and SZMC 25875 resulted in significant inhibition compared to the positive control ($p<0.05$), while 25 and 50% CCF of *P.*

resinovorans SZMC 25872 led to a significant suppression of *A. tumefaciens* compared to all the positive control and the inhibition caused by the negative control and other strains ($p<0.05$), regardless of the presence or absence of ascorbic acid. Vitamin C did not eliminate either the significant moderate suppression ($p<0.05$) caused by the negative control and the CCF of *E. adhaerens* SZMC 25856, as well as the significant, almost complete inhibition of *A. tumefaciens* SZMC 14557 ($p<0.05$) by *P. resinovorans* SZMC 25872 and SZMC 25875, obtained from MM+L-alanine (Figure 13).

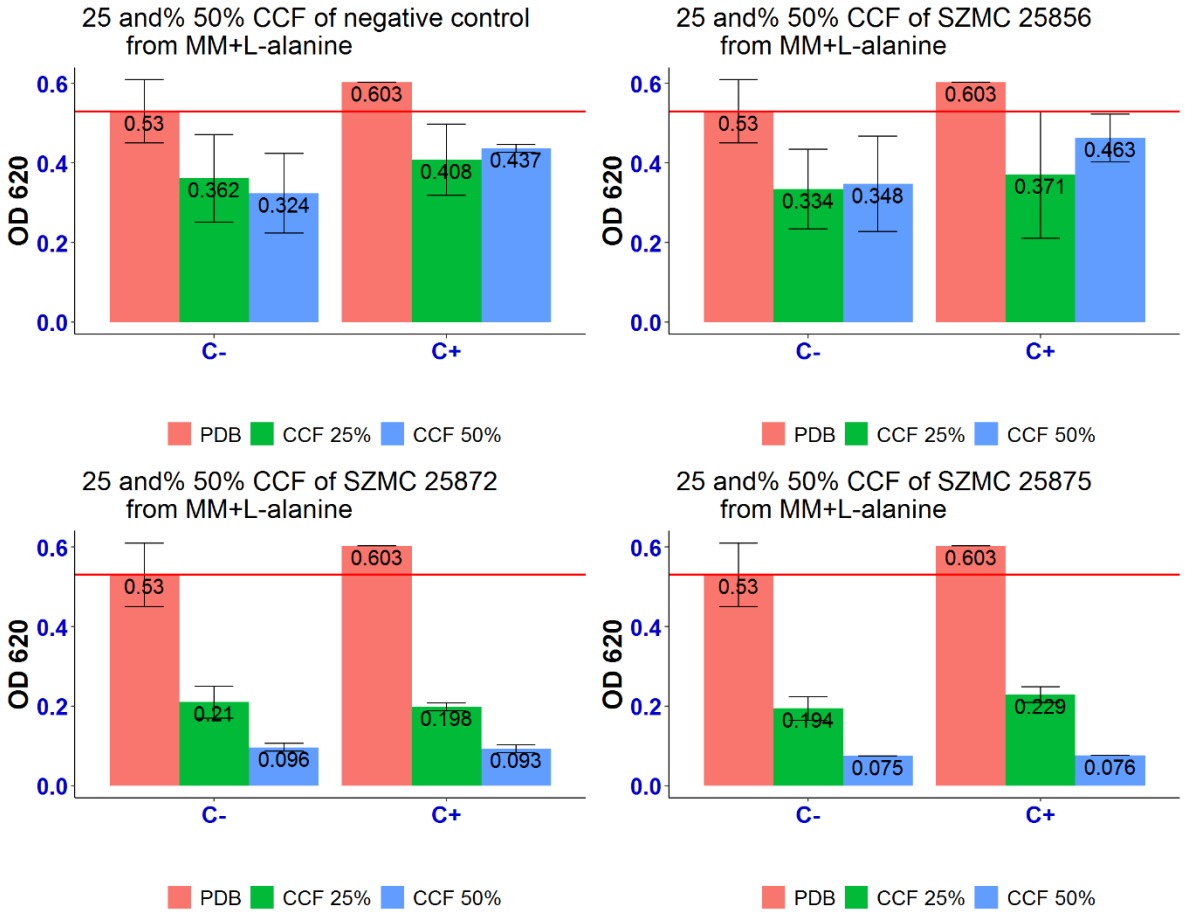


Figure 13. The effect of 25 and 50% CCF of the control (non-inoculated medium) and 3 glyphosate-tolerant bacteria obtained from MM amended with L-alanine on the growth of *A. tumefaciens* SZMC 14557 in the absence (C-) and presence (C+) of 1 mM ascorbic acid. PDB is positive control (no added CCF), CCF 25% and CCF 50% are the corresponding CCF concentrations. The red line represents the lowest value of the positive control.

Despite the significant growth promotion in some cases, the addition of 1 mM ascorbic acid did not eliminate the growth inhibition of *A. tumefaciens* in samples treated with CCF of either MM+succinic acid or L-alanine. Therefore, based on our findings, it can be suggested that the inhibition mode by CCF from either MM+succinic acid or MM+L-alanine does not include ROS generation.

The thermal stability of the potential active metabolites was evaluated after heat-treetment of CCF at 80 °C for 60 min and 90 °C for 30 min. The first treatment at lower temperature did not cause remarkable difference in the inhibition rate between heated and non-heated samples (Table 17).

Table 17. The effect of non-heated and heated (80 °C for 60 min) CCF obtained from MM+succinic acid and MM+L-alanine on the growth of *A. tumefaciens* (OD_{620}, 24-h incubation)

Samples	25% CCF		50% CCF	
	Non-heated	Heated	Non-heated	Heated
MM+L-alanine				
PDB	0.484±0.04			
Control	0.298±0.01	0.324±0.03	0.267±0.00	0.276±0.02
SZMC 25856	0.343±0.02	0.350±0.00	0.297±0.03	0.312±0.01
SZMC 25872	0.230±0.04	0.216±0.01	0.135±0.01	0.135±0.01
SZMC 25875	0.238±0.02	0.226±0.01	0.112±0.02	0.119±0.01
MM+succinic acid				
PDB	0.494±0.03			
Control	0.377±0.01	0.409±0.04	0.344±0.00	0.370±0.02
SZMC 25856	0.394±0.01	0.391±0.00	0.369±0.01	0.370±0.02
SZMC 25872	0.360±0.03	0.372±0.00	0.240±0.02	0.270±0.01
SZMC 25875	0.365±0.02	0.350±0.00	0.367±0.01	0.361±0.01

The inhibition pattern in both heated and non-heated samples was the same as in the previous assays: significant inhibition compared to positive control as well as to CCF of non-inoculated medium and SZMC 25856 was observed in the cases of SZMC 25872 at 50% succinic acid and of both SZMC 25872 and SZMC 25875 at 25 and 50% of L-alanine, respectively ($p<0.05$). However, reheating of the samples at 90 °C for 30 min resulted in a non-significant ($p>0.05$) but a certain decrease of the inhibition caused by 50% heated CCF of SZMC 25872 from succinic acid compared to non-heated samples (Table 18).

Table 18. The effect of non-heated and heated (90 °C for 30 min) CCF obtained from MM+succinic acid and MM+L-alanine on the growth of *A. tumefaciens* (OD_{620}, 24-h incubation)

Samples	25% CCF		50% CCF	
	Non-heated	Heated	Non-heated	Heated
MM+L-alanine				
PDB	0.619±0.03			
Control	0.383±0.02	0.414±0.02	0.319±0.01	0.403±0.08
SZMC 25856	0.364±0.02	0.410±0.01	0.325±0.02	0.363±0.01
SZMC 25872	0.203±0.02	0.221±0.02	0.108±0.01	0.110±0.00
SZMC 25875	0.220±0.02	0.220±0.02	0.078±0.01	0.081±0.01
MM+succinic acid				
PDB	0.617±0.04			
Control	0.426±0.01	0.420±0.01	0.382±0.02	0.387±0.03
SZMC 25856	0.407±0.02	0.418±0.01	0.376±0.01	0.376±0.01
SZMC 25872	0.494±0.12	0.468±0.07	0.283±0.06	0.385±0.04
SZMC 25875	0.440±0.01	0.424±0.04	0.439±0.02	0.411±0.02

Sakr et al. (2013) reported the total inhibition of lactonase activity treated at either 80 or 90 °C. Broadly assuming that lactonase and metabolic compounds detected in succinic acid (Subsection 6.4.9) share similar thermal characterisitcs (because heating at 90 °C resulted in the elimination of the lactonase activity and in the mitigation of the inhibition by CCF from succinic acid), it might be supposed that these identified metabolites are involved in the inhibition of *A. tumefaciens*. Furthermore, no changes of the inhibition rate between

heated and non-heated samples were found in the case of L-alanine containing no identified metabolites with similar composition to homoserine lactones. Therefore, these results indicated the similar thermal stability of lactonase and metabolites detected in CCF of SZMC 25872 from succinic acid and their possible inhibitory effect.

6.4.8 Extracellular enzyme activity assays

Certain extracellular enzymes, such as chitinases, cellulases, proteases, and β-glucanases take part not only in the elimination of plant pathogens, but can also indirectly stimulate plant growth (Mishra et al. 2020). Therefore, the strains tested in the biocontrol studies (Subsection 6.4) were examined for their trypsin and chymotrypsin-like protease, as well as esterase activities in 7 different media (Figure 14). Among the selected glyphosate-tolerant strains, *P. resinovorans* (genotype I) SZMC 25872 (the most promising biocontrol agent) showed the most intensive activity of all 3 tested enzymes in all 7 media with the exception of MM amended with glucose, while *E. adhaerens* SZMC 25856 and *P. resinovorans* (genotype II) SZMC 25875 showed substantial enzyme activities only in the presence of sodium pyruvate and L-alanine as carbon sources. Among the studied carbon sources, sodium pyruvate stimulated the activity of all 3 enzymes of all 3 tested strains, while L-alanine also resulted in high enzyme activities of *E. adhaerens* SZMC 25856 and *P. resinovorans* (genotype I) SZMC 25872. No enzyme activity for any of the studied strains was detected in MM amended with glucose.

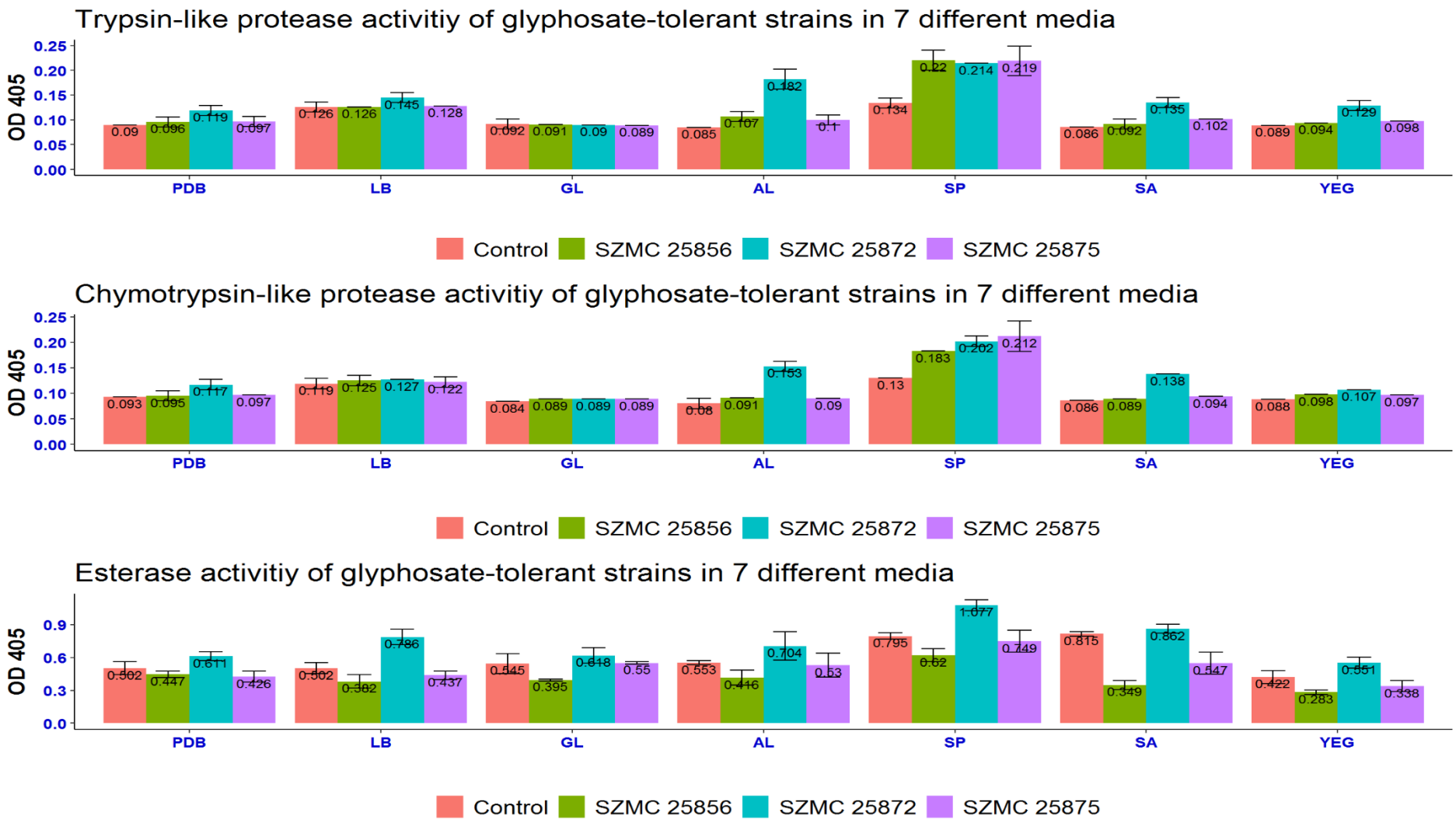


Figure 14. Extracellular enzyme activities of 3 glyphosate-tolerant bacteria, *E. adhaerens* SZMC 25856, *P. resinovorans* (genotype I) SZMC 25872, and *P. resinovorans* (genotype II) SZMC 25875 grown in different media, control is negative control (non-inoculated media). PDB, LB, GL, AL, SP, SA, and YEG are media: PDB, LB, MM+glucose, MM+L-alanine, MM+sodium pyruvate, MM+succinic acid, and YEG, respectively.

The highest activities of *P. resinovorans* SZMC 25872 of both trypsin- and chymotrypsin-like proteases were found in MM amended with sodium pyruvate and L-alanine as carbon source. Therefore, based on these results, it can be suggested that extracellular protease activities might be involved in the inhibition of *A. tumefaciens*, particularly in the case of MM+L-alanine, as 25 and 50% CCF obtained from these media inhibited significantly *A. tumefaciens* SZMC 14557 (Subsection 6.4.5). Regarding esterases, activities could also be detected only in the case of *P. resinovorans* SZMC 25872, particularly when the strain was grown in LB and MM+sodium pyruvate. Because the highest esterase activity was found in CCF of LB, and none of the CCF obtained from this medium appeared to suppress *A. tumefaciens* (Subsection 6.4.5), it might be concluded that esterases do not play role in the inhibitory effect.

The secretion of lytic enzymes by rhizosphere-inhabiting microbes is known as an efficient response to combat the phytopathogens via direct inhibition or competition for space and nutrients (Vörös et al. 2019; Mishra et al. 2020). Extracellular hydrolytic enzymes, such as chitinases, proteases, and β-1,3-glucanases produced by *Bacillus*, *Pseudomonas*, *Trichoderma* strains were reported to have the inhibitory effect on a variety of plant pathogens, including *F. culmorum, F. oxysporum, F. solani, X. campestris* pv. *glycines*, and *R. solani* (Mishra et al. 2020). The antagonistic strains *B. amyloliquefaciens* SZMC 6161J and SZMC 6225J, *B. mojavensis* SZMC 6168J, and *B. subtilis* SZMC 6179J were reported to secrete chymotrypsin- and trypsin-like protease, as well as lipases enzymes (Vágvölgyi et al. 2013). Chitinases were also reported to trigger plant defence mechanisms and to have potential in insect control (Gomaa 2021).

6.4.9 MS-MS analysis and identification of the bioactive metabolites potentially involved in the biocontrol of *A. tumefaciens*

According to the analysis of the HPLC-HRMS data, none of the known inhibitory agents was identified in the supernatant obtained from MM amended with either succinic acid or L-alanine. However, several detected potential active metabolites are listed in Table 19.

Table 19. Identified metabolites with the potential to inhibit of *A. tumefaciens*

Name	Molecular ion	RT, min	MW	Media	Producer strain
Compound 1	$[M-H]^-$	7.91	207.0755	AL	SZMC 25875
Compound 2	$[M-H]^-$	7.69	261.0247	AL	SZMC 25875
Compound 3	$[M-H]^-$	15.57	385.2826	AL	SZMC 25875
Compound 4	$[M-H]^-$, $[M+H]^+$	17.22	299.2458	AL	SZMC 25872
Compound 5	$[M-H]^-$	17.44	325.2615	AL	SZMC 25875
Compound 6	$[M-H]^-$, $[M+H]^+$	16.56	369.2876	AL	SZMC 25875
Compound 7	$[M-H]^-$	7.68	337.0103	AL	SZMC 25872
Compound 8	$[M-H]^-$	7.69	338.9682	AL	SZMC 25872
Compound 9	$[M-H]^-$	4.34	346.0137	AL	SZMC 25875
Compound 10	$[M-H]^-$	3.37	346.0137	AL	SZMC 25875
Compound 11	$[M-H]^-$	7.68	358.9922	AL	SZMC 25875
Compound 12	$[M-H]^-$	6.33	373.0676	AL	SZMC 25875
Compound 13[a]	$[M+H]^+$	8.93	171.0898	SA	SZMC 25872
Compound 14[a]	$[M+H]^+$	9.32	171.0898	SA	SZMC 25872
Compound 15[a]	$[M+H]^+$	8.68	171.0898	SA	SZMC 25872

Compound 16[a]	$[M+H]^+$	9.71	171.0898	SA	SZMC 25872
Compound 17[b]	$[M+H]^+$	11.89	199.1208	SA	SZMC 25872
Compound 18[b]	$[M+H]^+$	11.01	199.1208	SA	SZMC 25872
Compound 19[b]	$[M+H]^+$	11.58	199.1208	SA	SZMC 25872
Compound 20[c]	$[M+H]^+$	12.24	213.1365	SA	SZMC 25872
Compound 21[c]	$[M+H]^+$	12.92	213.1365	SA	SZMC 25872
Compound 22	$[M-H]^-$	5.96	275.1004	SA	SZMC 25872
Compound 23	$[M-H]^-$	8.99	405.0480	SA	SZMC 25872
Compound 24	$[M-H]^-$	8.98	374.1186	SA	SZMC 25872

a,b,c, According to their fragmentation pattern they belong to the same compound class.

The identification of the secondary metabolites was performed based on exact mass. The exact mass of all the found metabolites was compared to those presented in 3 different own in-house secondary metabolite databases involving all the published secondary metabolites of the *Pseudomonas* genus. Furthermore, two literature surveys were performed to find the most relevant compounds. The first survey included relevant references found by keywords "*Agrobacterium tumefaciens*" and "biocontrol". The aim of this search was to find all metabolites produced by different microbial species that were reported to have the inhibitory effect on *Agrobacterium* species (relevant studies are summarised in Table 2). The second survey included appropriate references found by keywords "*Pseudomonas aeruginosa*" and "biocontrol". *P. aeruginosa* was selected because *P. resinovorans* is phylogenetically closely related to this species, moreover, *P. aeruginosa* is one of the-most widely studied species, and a variety of its metabolites, including active compounds has been reported so far. Bioactive compounds produced by certain *Pseudomonas* species, such as *P. protegens, P. fluorescens,* etc. were also included. The aim was to find all metabolites produced by *Pseudomonas* species that are reported to have the inhibitory effect on plant pathogens (relevant studies are summarised in Table 20).

Several compounds, such as (13, 14, 15, 16), (17, 18, 19) as well as (20 and 21) detected in succinic medium can be classified as the same chemical class as they share the same molecular weigth (MW) (Table 19) and fragmentation pattern. Furthermore, all mentioned compounds have similar composition to homoserine lactones (N-butyryl-L-homoserine-lactone, N-hexanoyl-L-Homoserine lactone, and N-heptanoyl-homoserine-lactone), respectively, but *m/z* rato of their main fragments was different from the known lactones which posses 102.0555 *m/z* as the main fragment (Girard et al. 2017). They might also share similar thermal characteristics with lactonase because after heat treatment at 90 °C for 30 min, both activity of lactonase from *B. weihenstephanensis* P65 (Sakr et al. 2013) and the inhibitory effect by succinic acid was eliminated or mitigated, respectively (Subsection 6.4.7). Compounds 22, 23, and 24 also detected in succinic acid only might act as siderophore compounds, because the inhibitory effect by succinic acid was eliminated in the presence of an exta iron source (Subsection 6.4.7). None of these 24 compounds were identified in either available databases or relevant references, except for 9 compounds, which, as it was mentioned, possess similar chemical structure to lactones (based on the mass list of own in-house database), yet they are not lactones as they differ by the main fragments.

Molina et al. (2003) reported the inhibition of *A. tumefaciens* via the degradation of their AHL, while Uroz and Heinonsalo (2008) also found no QS activities of *A. tumefaciens* treated with the supernatant having the AHL-deleterating potential. Tay and Yew (2013) summarised several QS inhibitors, (including characterised and new disruptors). They mentioned that QS is species-specific system and more than one system can be applied by bacteria and thus, combintation of the inhibitory techniques is necessary in some cases to inhibit bacterial growth. AHLs inhibited hemolytic activity and the growth of *S. pyogenes* M6

S165, as well as increased the influx of iron in the cells and interfered with several genes in the studies by Saroj et al. (2017). Therefore, both siderophores (limiting avaiable iron in the growth medium) and metabolitc compounds detected in succinic acid (increasing bacterial needs for iron and intefere with the gene expression and QS activity) might play an assemled role in the inhibition of *A. tumefaciens*.

The total ion chromatogram and MS-MS spectrum of the certain compounds produced by both SZMC 25872 and SZMC 25875 strains (12 and 24, detected only in L-alanine or succinic acid, respectively) are presented in Figures 15 and 16.

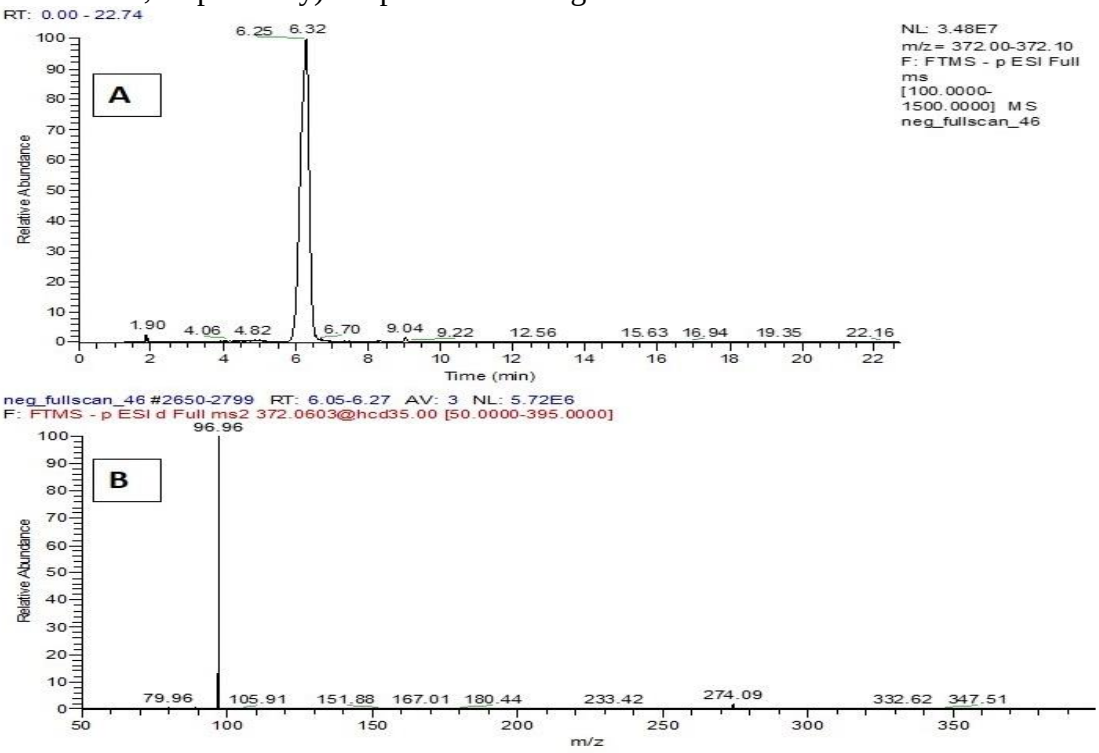


Figure 15. Total ion chromatogram (A) and MS-MS spectrum (B) of compound 12

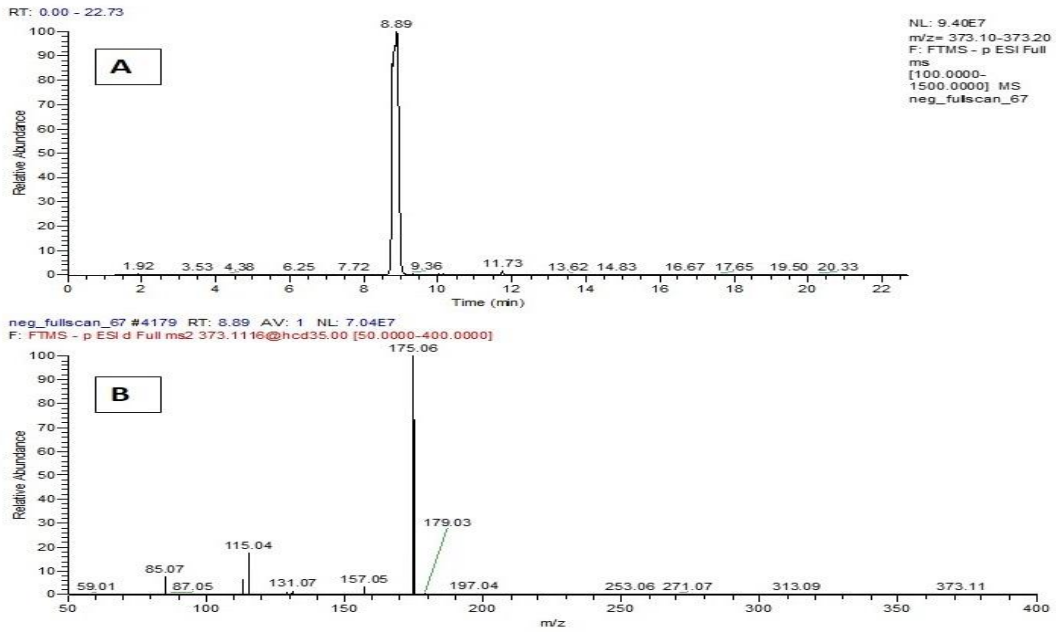


Figure 16. Total ion chromatogram (A) and MS-MS mass spectrum (B) of compound 24

The genus *Pseudomonas* involves various species with the ability of producing different types of metabolites active against a range of plant pathogens, some well-characterised examples are presented in Table 20. However, as *P. resinovorans* is not reported as potential biocontrol species yet, no information about its metabolites is avaibale in the literature and detected compounds might represent novel class of the inhibitory agents active against *A. tumefaciens*. Further investigations are needed to verify exact metabolites and their inhibitory potential.

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Table 20. Metabolites produced by different *Pseudomonas* strains with antimicrobial activity towards plant pathogens.

Antagonized organisms	Active compound	Producing strains	Reference
Phenazines			
F. graminearum	phenazine-1-carboxamide	*P. aeruginosa* NF011	Sun et al. 2021
Ganoderma boninense	phenazine	*P. aeruginosa* UPMP3	Parvin et al. 2020
R. solani, X. oryzae pv. *oryzae*	phenazine-1-carboxamide	*P. aeruginosa* MML2212	Shanmugaiah et al. 2009
Macrophomina phaseolina	phenazine-1-carboxylic acid	*P. aeruginosa* GS-33	Patil et al. 2016
R. solani	phenazine like compound	*P. aeruginosa* VSMKU1	Karmegham et al. 2020
R. solani, P. ultimum, F. oxysporum	phenazines	*P. chlororaphis* ssp. *aureofaciens* DSM 6698	Mezaache-Aichour et al. 2013
M. phaseolina, Dreschlera graminae	phenazine	*P. aeruginosa* RM-3	Minaxi and Saxena 2010
F. oxysporum f. sp. *ciceris, F. udum*	phenazine-1-carboxylic acid, oxychlororaphin	*P. aeruginosa* PNA1	Anjaiah et al. 2003
A. alternata, A. solani, B. australiensis, C. acutatum, C. andropogonis, F. oxysporum, F. moniliforme, P. aphanidermatum, R. solani	1-hydroxyphenazine	*P. aeruginosa* SD12	Dharni et al. 2012
Siderophores			
F. oxysporum f. sp. *ciceri, F. udum, Aspergillus niger*	siderophores (hydroxamate type)	*P. aeruginosa* JAS-25	Sulochana et al. 2014
X. campestris pv. *campestris*	4-hydroxy-2-nonyl-quinoline	*P. aeruginosa* KA19	Mishra and Arora 2012
X. citri ssp. *citri*.	enantio-pyochelin	*P. protegens* CS1	Michavila et al. 2017
E. carotovora	pseudobactin	*Pseudomonas* sp. B10	Ambrosi et al. 2000
Verticillium dahliae	7-hydroxytropolone (7-HT)	*P. protegens* XY2F4	Tao et al. 2020
Antibiotics and lipids			
F. oxysporum, F. moniliforme, R. solani, C. gloeosporioides, C. falcatum, A. niger, A. flavus	iturin, surfactins, fengycin, diacetylphloroglucinol (DAPG), phenazine	*Pseudomonas* and *Bacillus* species	Ali et al. 2020
P. grisea, G. graminis, F. oxysporum, X. campestris, E. persicina	2,4-DAPG	*P. putida, P. aeruginosa*	Sekar and Prabavathy 2014
Rhizopus microsporus, F. oxysporum, A.	pyrrolnitrin	*P. aeruginosa* PS24	Uzair et al. 2018

niger, A. alternata, Penicillium digitatum			
F. oxysporum f. sp. *cubense*	2,4-DAPG	*P. aeruginosa* FP10	Ayyadurai et al. 2006
F. oxysporum f. sp. *radicis-cucumerinum*	4-DAPG	*P. aeruginosa* P23	Bradley and Punja 2010
M. phaseolina, F. oxysporium, P. nicotianae	rhamnolipid	*P. aeruginosa* DR1	Reddy et al. 2016
X. campestris, F. solani, C. invisium	dirhamnolipid	*P. aeruginosa* RTE4	Chopra et al. 2020
F. verticillioides	rhamnolipids	*P. aeruginosa* SS14	Borah et al. 2016
F. proliferatum NCIM 1105, *A. niger* NCIM 596	rhamnolipid	*P. aeruginosa* CPCL	Arutchelvi and Doble 2010
Other compounds			
phytopathogenic *Xanthomonas* and *Pseudomonas* strains	tailocins	*P. fluorescens* SF4c	Fernandez et al. 2017
Sporisorium scitamineum, Ceratocystis paradoxa, F. verticillioides	hydrogen cyanide, salicylate, chitinase activity, exopolysaccharides	*P. aeruginosa* B18	Singh et al. 2021
M. phaseolina, Fusarium sp., *Rhizoctonia* sp. *Alternaria* sp., *Aspergillus* sp.	3,4-dihydroxy-N-methyl-4-(4-oxochroman-2-yl)butanamide	*P. aeruginosa* PGPR2	Illakkiam et al. 2013
E. amylovora	L-2-amino-4-methoxy-*trans*-3-butenoic acid	*P. aeruginosa*	Lee et al. 2013
M. phaseolina	purified exopolysaccharides	*P. aeruginosa* PF23	Tewari and Arora 2014
Phytophthora palmivora	partially purified proteins	*P. aeruginosa* RS1	Sowanpreecha and Rerngsamran 2018
M. phaseolina	7-hydroxytropolone (7HT)	*P. donghuensis* SVBP6	Muzio et al. 2020

6.5 Eco-physiological characterisation of glyphosate-tolerant bacteria

6.5.1 pH spectrum testing

The growth of the isolates was tested at pH values ranging between 6.09 and 8.95. All the 3 examined glyphosate-tolerant strains, regardless of the species, were found to prefer neutral and alkaline pH (6.59–7.96) (Figure 17).

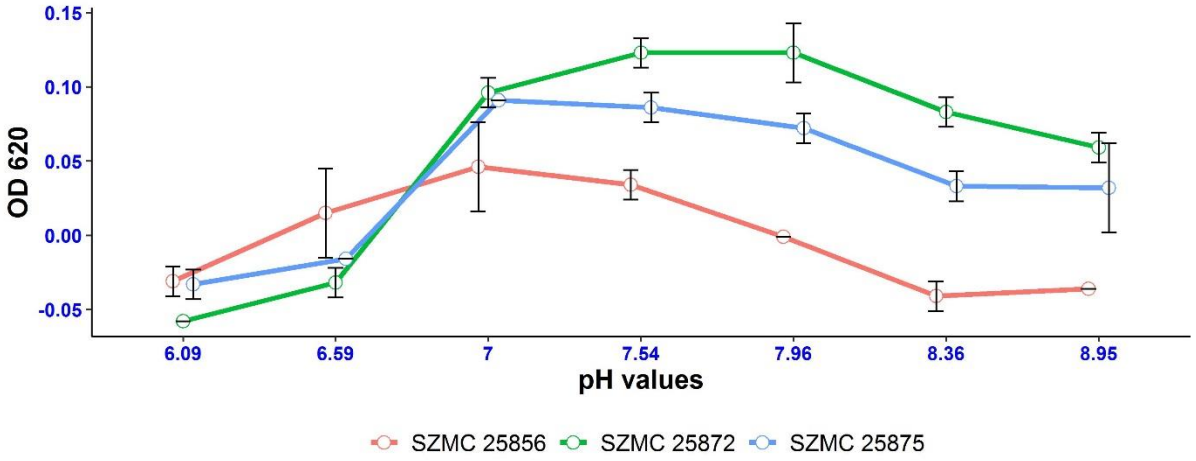

Figure 17. The effect of pH on the growth of glyphosate-tolerant bacteria, *E. adhaerens* SZMC 25856, *P. resinovorans* (genotype I) SZMC 25872, and *P. resinovorans* (genotype II) SZMC 25875.

As certain microorganisms are viable in just a relatively narrow pH range, soil pH has a significant impact on the diversity of soil-inhabiting bacteria (Zheng et al. 2019; Wang et al. 2019). In our study, all glyphosate-tolerant isolates showed sufficient growth in the pH range of 6.59-7.96 (Figure 17), suggesting that neutral and slightly alkaline soils could be optimal for their growth. *E. meliloti* LMG 6133 and *E. medicae* LMG 19920 grew in the pH range of 5.5-8.5 (Alías-Villegas et al. 2015), and the optimal pH value for *E. adhaerens* OV14 was found to be 6.5 (Rathore et al. 2015). In contrast, *P. resinovorans* SPR1 was reported to tolerate a wide pH range, from 4 to 11 (Ashengroph et al. 2011). *P. aeruginosa* GS-33 could also grow in the pH range of 4-11, but the optimum was found to be pH 8 (Patil et al. 2016).

Based on our findings, the optimal pH range for the successful application of the glyphosate-tolerant bacteria *in vivo* pH level might vary from 6.5 to 8.0.

6.5.2 Heavy metal tolerance assay

E. adhaerens SZMC 25856 showed a broad-scale tolerance to heavy metals, as its growth was not inhibited significantly by most of the studied heavy metals at a concentration of 0.1 mM ($p>0.05$), except for Hg and Cr (Figure 18). Cr, and Ni ions significantly inhibited the growth of *P. resinovorans* SZMC 25875 ($p<0.05$), while SZMC 25872 was found to be the most sensitive, as it was inhibited by 0.1 mM Al, Zn, Cr, Hg, and Cd. However, all isolates showed substantial growth in the presence of 0.1 mM of the remaining heavy metals without significant inhibition compared to the positive control ($p>0.05$).

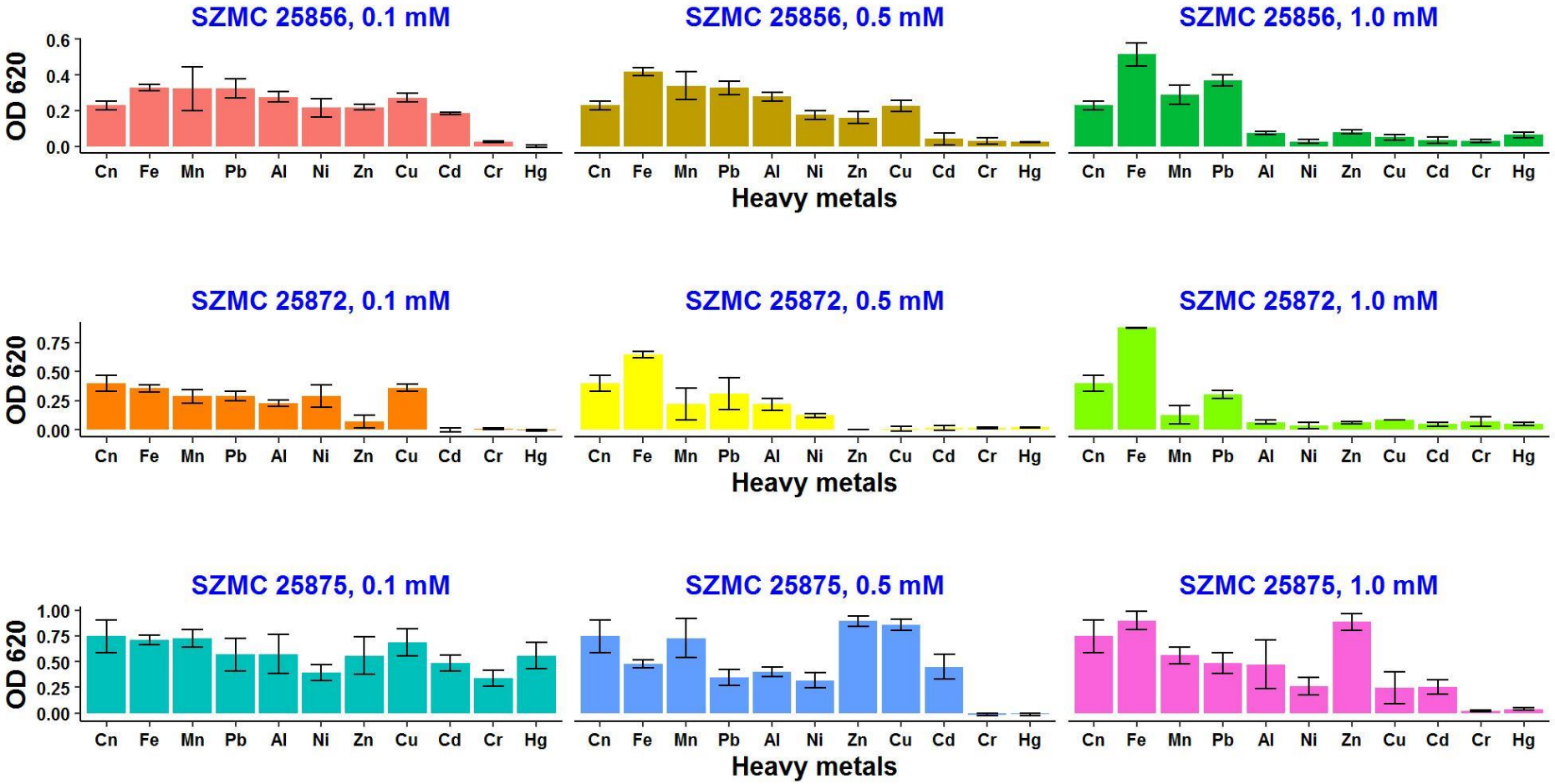

Figure 18. The effect of heavy metal ions (0.1-1.0 mM) on the growth of glyphosate-tolerant bacteria, *E. adhaerens* SZMC 25856, *P. resinovorans* (genotype I) SZMC 25872, and *P. resinovorans* (genotype II) SZMC 25875.

All glyphosate-tolerant strains showed considerable tolerance to the tested heavy metals at 0.1 mM, however, increasing the concentration to 0.5 and 1.0 mM resulted in reduced viability. The growth of *E. adhaerens* SZMC 25856 was significantly inhibited by 0.5 mM Hg, Cd, Zn, and Cr ($p<0.05$) (Figure 18). The majority of the tested heavy metals, except for Fe, Mn, and Pb resulted in a significantly decreased growth of *P. resinovorans* (genotype I) SZMC 25872 ($p<0.05$), after the application of 0.5 mM, but (genotype II) SZMC 25875, could grow in the presence of Cu, Al, Zn, Fe, Mn, Pb, and Cd ($p>0.05$) used in same higher concentration.

Testing the effect of heavy metals at 1.0 mM revealed that *E. adhaerens* SZMC 25856 strain could grow in the presence of Fe, Mn, and Pb, but *P. resinovorans* (genotype I) SZMC 25872 was tolerant only to Fe and Pb (no significant changes between the control and metal-containing samples were observed, $p>0.05$) (Figure 18). *P. resinovorans* (genotype II) SZMC 25875 was able to grow in the presence of 1.0 mM Zn, Al, Fe, Mn, and Pb ($p>0.05$).

Interestingly, the presence of Fe at 0.1-1.0 mM concentration significantly stimulated the growth of *E. adhaerens* SZMC 25856 and 0.5-1.0 mM had positive influence on *P. resinovorans* SZMC 25872 ($p<0.05$). These strains produced remarkably high amounts of siderophores (Table 16, Subsection 6.4.6), therefore, it might be concluded that iron might play role in the metabolism of these isolates. Furthermore, 1.0 mM Pb also positively affected the growth of *E. adhaerens* ($p<0.05$).

Different industrial and agricultural practices resulted in an increased accumulation of toxic heavy metals in different areas (Lutts and Lefèvre 2015). Besides the well-known deleterious effects on ecosystems and human health (Jaiswal et al. 2018), heavy metals possess a serious obstacle for the biodegradation of pollutants via the inhibition of the growth of bacteria (Chu 2018) including pesticide-degrading species (Olaniran et al. 2013, Zhang et al. 2020). Therefore, as polluted agricultural soils might contain heavy metals at concentrations inhibitory to microbial growth, it is important to test the metal tolerance of beneficial inoculants before their field application. In our study, *P. resinovorans* and *E. adhaerens* isolates were tested for their tolerance to 10 different heavy metal ions at 0.1, 0.5, and 1.0 mM, as they, particularly Cu, Mn, Ni, Fe, Zn, and Cd, may be present in agricultural soils at these concentrations (Vörös et al. 2019). Our findings in comparison with previously published data are summarised in Table 21.

Table 21. Heavy metal tolerance of different bacterial strains

Heavy metal	Strain	Highest tolerated concentration	Reference
Cu	***E. adhaerens* SZMC 25856**	0.5 mM	Present study
	E. adhaerens OS3	12.59 mM (800 µg/ml*)	Oves et al. 2017
	***P. resinovorans* SZMC 25872**	0.1 mM	Present study
	***P. resinovorans* SZMC 25875**	0.5 mM	Present study
	P. moraviensis	0.4 mM (100 ppm*)	Hassan et al. 2017
	P. aeruginosa S6	1.2 mM	Hassen et al. 1998
	B. velezensis 6161J	0.1 mM	Vörös et al. 2019
Zn	***E. adhaerens* SZMC 25856**	0.1 mM	Present study
	E. adhaerens OS3	12.24 mM (800 µg/ml*)	Oves et al. 2017
	E. meliloti LMG 6133	1.25 mM	Alías-Villegas et al. 2015
	***P. resinovorans* SZMC 25872**	<0.1 mM	Present study
	***P. resinovorans* SZMC 25875**	1.0 mM	Present study
	P. aeruginosa S6	1.5 mM	Hassen et al. 1998

	B. velezensis 6161J	< 0.1 mM	Vörös et al. 2019
Ni	***E. adhaerens* SZMC 25856**	0.5 mM	Present study
	E. adhaerens OS3	17.04 mM (1000 µg/ml)	Oves et al. 2017
	***P. resinovorans* SZMC 25872**	0.1 mM	Present study
	***P. resinovorans* SZMC 25875**	0.1 mM	Present study
	P. moraviensis	0.39 mM (50 ppm*)	Hassan et al. 2017
	B. velezensis 6161J	0.1 mM	Vörös et al. 2019
Hg	***E. adhaerens* SZMC 25856**	< 0.1 mM	Present study
	E. medicae M-7C, J-7C	25 µM	Ruiz-Díez et al. 2012
	***P. resinovorans* SZMC 25872**	<0.1 mM	Present study
	***P. resinovorans* SZMC 25875**	0.1 mM	Present study
	P. aeruginosa S6	0.08 mM	Hassen et al. 1998
Pb	***E. adhaerens* SZMC 25856**	1.0 mM	Present study
	***P. resinovorans* SZMC 25872**	1.0 mM	Present study
	***P. resinovorans* SZMC 25875**	1.0 mM	Present study
	P. moraviensis	0.15 mM (50 ppm*)	Hassan et al. 2017
Fe	***E. adhaerens* SZMC 25856**	1.0 mM	Present study
	***P. resinovorans* SZMC 25872**	1.0 mM	Present study
	***P. resinovorans* SZMC 25875**	1.0 mM	Present study
	B. velezensis 6161J	1.0 mM	Vörös et al. 2019
Mn	***E. adhaerens* SZMC 25856**	1.0 mM	Present study
	***P. resinovorans* SZMC 25872**	1.0 mM	Present study
	***P. resinovorans* SZMC 25875**	1.0 mM	Present study
	P. moraviensis	0.79 mM (100 ppm*)	Hassan et al. 2017
	B. velezensis 6161J	1.0 mM	Vörös et al. 2019
Cd	***E. adhaerens* SZMC 25856**	0.1 mM	Present study
	E. adhaerens OS3	2.22 mM (250 µg/ml)	Oves et al. 2017
	E. meliloti LMG 6133	0.25 mM	Alías-Villegas et al. 2015
	***P. resinovorans* SZMC 25872**	<0.1 mM	Present study
	***P. resinovorans* SZMC 25875**	0.5 mM	Present study
	P. moraviensis	0.27 mM (50 ppm*)	Hassan et al. 2017
	P. aeruginosa S6	1.5 mM	Hassen et al. 1998
	B. velezensis 6161J	< 0.1 mM	Vörös et al. 2019
Cr	***E. adhaerens* SZMC 25856**	< 0.1 mM	Present study
	E. adhaerens OS3	9.61 mM (500 µg/ml*)	Oves et al. 2017
	***P. resinovorans* SZMC 25872**	<0.1 mM	Present study
	***P. resinovorans* SZMC 25875**	0.1 mM	Present study
	P. moraviensis	0.38 mM (100 ppm*)	Hassan et al. 2017
	P. aeruginosa S6	1.2 mM	Hassen et al. 1998

Strains isolated in the present study are set in bold.

*As reported in the cited reference.

The *P. moraviensis* strain evaluated by Hassan et al. (2017) could tolerate 0.4 mM Cu, 0.79 mM Mn, and 0.38 mM Cr, as well as 0.39 mM Ni, 0.15 mM Pb, and 0.27 mM Cd. The promising biocontrol strain *B. velezensis* SZMC 6161J grew in the presence of 1.0 mM Fe and Mn as well as 0.1 mM Cu and Ni, but 0.1 mM Cd and Zn suppressed its growth (Vörös et al. 2019). The minimum inhibitory concentrations of Cu and Cd for *P. aeruginosa* S6 were

1.2 and 1.5 mM, respectively (Hassen et al. 1998). In our study, *P. resinovorans* SZMC 25872 and SZMC 25875 and *E. adhaerens* SZMC 25856 tolerated 1.0 mM Fe, Pb, and Mn, while Fe even significantly promoted *E. adhaerens* and *P. resinovorans* SZMC 25872 (Figure 18). This tolerance can be explained by the presence of several heavy metal transporting ATPase, such as Pb-Cd-Zn-Hg transporting ATPase identifed in the whole genome of all the 3 tested strains (Section 6.6), which play role in the elimination of the toxic effect of these heavy metals (Argüello et al. 2011). *E. adhaerens* SZMC 25856 was also tolerant to Zn at 0.1 mM, while at 0.5 mM concentration to Cu and Ni (Figure 18). Oves et al. (2017) reported *E. adhaerens* OS3 to tolerate 2.22 mM Cd, 9.61 mM Cr, 12.59 mM Cu, 12.24 mM Zn, and 17.04 mM Ni, and due to its biosorption abilities, this strain was proposed as a promising species to clean up heavy metal-polluted substrates. *E. meliloti* LMG 6133 isolated from *Medicago marina* was resistant to 0.25 mM Cd and 1.25 mM Zn (Alías-Villegas et al. 2015). Ruiz-Díez et al. (2012) reported that the minimum inhibitory concentrations of Hg for 7 *E. medicae* strains recovered from mercury-contaminated soils appeared to fall in the range of 3-12.5 µM, and the most resistant M-7C and J-7C isolates survived in the presence of 25 µM Hg. The growth of *E. adhaerens* SZMC 25856 and *P. resinovorans* (genotype I) SZMC 25872 was completely inhibited by 0.1 mM Hg in our study, but *P. resinovorans* (genotype II) SZMC 25875 could tolerate this concentration (Figure 18). *P. aeruginosa* S6 resisted 1.2 mM Cr, 0.4 mM Co, 1.5 mM Zn, and 0.08 mM Hg (Hassen et al. 1998). The susceptibility of bacteria to heavy metals depends on various factors, including the distinct compounds, their availability and concentrations, as well as environmental conditions (Igiri et al. 2018). As the *E. adhaerens* and *P. resinovorans* isolates used in this study could tolerate numerous heavy metals at different concentrations (0.1-1.0 mM), they might have the potential to be applied in heavy metal-polluted soils.

6.5.3 Pesticide sensitivity test

The effect of 33 different pesticides (18 herbicides, 14 fungicides and one insecticide with different modes of action and chemical structures) was tested on the growth of glyphosate-tolerant bacteria at 6.25, 12.5, and 25.0 µg/ml concentrations. *E. adhaerens* SZMC 25856 strain was able to grow in the presence of the insecticide diflubenzuron and all herbicides at the highest studied concentration without significant inhibition ($p>0.05$), except for propham, which was tolerated only at 6.25 µg/ml concentration (Table 22). Among the examined fungicides, only the highest concentration of captan, maneb, and zineb caused significant inhibition on the growth of *E. adhaerens* compared to the positive control ($p<0.05$). Imazalil, mancozeb, and thiram had the most deleterious effect on *E. adhaerens* SZMC 25856: imazalil inhibited its growth at 6.25 µg/ml, while mancozeb and thiram at 12.5 µg/ml concentration ($p<0.05$). The remaining fungicides did not suppress the growth of *E. adhaerens* significantly ($p>0.05$).

The *P. resinovorans* isolates of both genotypes could grow without significant inhibition between the positive control and treated samples ($p>0.05$) of most herbicides applied at 12.5-25.0 µg/ml concentration (Table 22). However, bensufuron-methyl and ethoxysulfuron inhibited the growth of SZMC 25875 at 25.0 and 12.5 µg/ml concentration ($p<0.05$), respectively, while SZMC 25872 was suppressed by 25.0 µg/ml ethoxysulfuron.

The fungicides maneb and mancozeb completely blocked the growth of both *P. resinovorans* strains even at the lowest studied concentration. Thiram did not cause significant growth suppression only at 6.25 µg/ml ($p>0.05$), while higher tested concentrations of these fungicides resulted in significant inhibition ($p<0.05$). The growth of *P. resinovorans* SZMC 25872 was also inhibited significantly by 12.5 µg/ml captan and zineb, whereas SZMC 25875 was suppressed by 6.25 and 25 µg/ml of the same fungicides

($p<0.05$). The inhibitory concentrations of thiram, as well as captan, maneb and zineb for *E. adhaerens* SZMC 25856 were 12.5 and 25 µg/ml, respectively ($p<0.05$). Imazalil inhibited the growth at the lowest studied concentration, while mancozeb suppressed bacterial growth at 12.5 µg/ml ($p<0.05$). The remaining pesticides, including the insecticide diflubenzuron did not cause remarkable change in the growth between the positive control and the treated samples ($p>0.05$).

Interestingly, several pesticides promoted bacterial growth significantly even at the highest concentration. In the case of *E. adhaerens*, the herbicides chlorotoluron, chlorsulfuron, diuron, primisulfuron-methyl at 25.0 µg/ml and glyphosate at 12.5-25.0 µg/ml, as well as the fungicide tebuconazole at 25 µg/ml positively affected its growth compared to the positive control ($p<0.05$). No significant increase due to the presence of pesticides was observed on *P. resinovorans* SZMC 25872 ($p>0.05$), while certain herbicides, such as chlorpropham, diuron, MCPA at 12.5-25.0 µg/ml as well as isoproturon and fenuron stimulated the growth of SZMC 25875 when applied at 25.0 µg/ml concentration ($p<0.05$). These pesticides share certain characteristics. All substances with stimulatory effect, except tebuconazole, are herbicides, and the majority of them (chlorotoluron, diuron, isoproturon, chlorsulfuron, primisulfuron methyl, and fenuron) represent the urea class (data from PPDB). Specifically, among the urea-based herbicides, chloroturon, diuron, isoproturon, and fenuron are phenylurea compounds, and they act as photosynthesis inhibitors. Chlorsulfuron and primisulfuron methyl are sulfonylurea herbicides, inhibiting the amino acid synthesis in plants (data from PPDB). Both phenylurea and sulfonylurea-type herbicides promoted *E. adhaerens* SZMC 25856, while only phenylurea herbicides stimulated the growth of *P. resinovorans* (genotype II) SZMC 25875. The tentative reason of the stimulatory effect of these herbicides can be the potential biodegradation of the examined glyphosate-tolerant strains. Various bacteria with the ability to tolerate high levels of pesticides have been identified as pesticide degraders (Shahid and Khan, 2022). Both *E. adhaerens* and *P. resinovorans* species were reported to have potential for the biodegradation of certain compounds: *E. adhaerens* - the insecticides thiamethoxam (Zhou et al. 2013), flonicamid (Zhao et al. 2021), and polychlorinated biphenyls (Xu et al. 2016); *P. resinovorans* – carbazole (Shintani et al. 2013) – industrial raw material for dyestuffs, medicines, and plastics, consumed as carbon and nitrogen source. Therefore, our strains might as well utilise the herbicides as additional nutrient source. These findings of pesticide tolerance and the tentative biodegradation potential are supported by the results of the subsequent genome sequence analysis, during which several enzymes playing role in xenobiotic tolerance or degradation were detected (Section 6.6). In particular, the enzymes benzoate 1,2-dioxygenase, quinone oxidoreductase, and catechol 1,2-dioxygenase were identified in all three glyphosate-tolerant strains. It is assumed that quinone oxidoreductase is partly involved in decreasing the level of free intracellular radicals, and the elimination of the toxicity of xenobiotics by the reduction of electrons, preventing the production of reactive semiquinones (Pey et al. 2019). Whereas, the potential of catechol 1,2-dioxygenases to remove various contamintants, including phenol, benzoate, chlorocatechol, the herbicide diuron, and polychlorinated biphenyls from wastewater has been reported (Setlhare et al. 2019). The presence of catechol 1,2-dioxygenases can be highlighted because they take part in the degradation of diuron (Selthare et al. 2019), and this herbicide was found to promote the growth of both *P. resinovorans* and *E. adhaerens.*

Table 22. The highest concentration (µg/ml) of pesticides tolerated by glyphosate-tolerant bacteria

Pesticide	*E. adhaerens* SZMC 25856	*P. resinovorans* (genotype I) SZMC 25872	*P. resinovorans*, (genotype II) SZMC 25875
Herbicides			
Bensulfuron-methyl	25.0	25.0	12.5
Chlorotoluron	25.0	25.0	25.0
Chlorpropham	25.0	25.0	25.0
Chlorsulfuron	25.0	25.0	25.0
Cinosulfuron	25.0	25.0	25.0
Diuron	25.0	25.0	25.0
Dimethachlor	25.0	25.0	25.0
Fenuron	25.0	25.0	25.0
Ethoxysulfuron	25.0	12.5	6.25
Glyphosate	25.0	25.0	25.0
Glyphosate (*Glialka Star*)	25.0	25.0	25.0
Isoproturon	25.0	25.0	25.0
Linuron	25.0	25.0	25.0
MCPA	25.0	25.0	25.0
Primisulfuron-methyl	25.0	25.0	25.0
Propham	6.25	25.0	25.0
Triasulfuron	25.0	25.0	25.0
2,4-D	25.0	25.0	25.0
Fungicides			
Captan	12.5	6.25	-
Carbendazim	25.0	25.0	25.0
Carboxin	25.0	25.0	25.0
Fenarimol	25.0	25.0	25.0
Flutriafol	25.0	25.0	25.0
Imazalil	-	25.0	25.0
Mancozeb	6.25	-	-
Maneb	12.5	-	-
Penconazole	25.0	25.0	25.0
Tebuconazole	25.0	25.0	25.0
Thiabendazole	25.0	25.0	25.0
Thiram	6.25	6.25	6.25
Thiophanate-methyl	25.0	25.0	25.0
Zineb	12.5	6.25	12.5
Insecticide			
Diflubenzuron	25.0	25.0	25.0

Pesticides and their derivates can have significant effect on different types of soil microorganisms inhabiting agricultural fields (Arora and Sahni 2016). Among the 33 tested pesticides, the carbamate-type fungicides thiram, zineb, maneb and mancozeb, as well as the sulfonylurea-based herbicides bensulfuron-methyl and ethoxysulfuron had the most deleterious effect on *P. resinovorans* strains of both genotypes and *E. adhaerens* SZMC 25856. Mancozeb significantly inhibited both fungal and bacterial populations, including actinomycetes, as well as nitrifying and ammonifying bacteria in soil samples from apple

orchards (Walia et al. 2014). According to the findings of Vörös et al. (2019), four fungicides, mancozeb, maneb, captan, and thiram suppressed the growth of the biocontrol strain *B. velezensis* SZMC 6161J even at the lowest tested concentration (6.25 µg/ml). In our studies, the herbicide diuron stimulated the growth of *P. resinovorans* SZMC 25875 at 12.5 and 25 µg/ml, and also promoted *E. adhaerens* SZMC 25856 at 25 µg/ml concentration. Vörös et al. (2019) also reported the lack of inhibition of diuron on the growth of *B. velezensis* SZMC 6161J. In contrast, according to the results of denaturing gradient gel electrophoresis (DGGE) analysis, the field application of diuron resulted in significant alterations of the indigenous microbiota during a 3-month period (Moretto et al. 2017), while the suppression of nitrogen-fixing bacteria was observed on the first day after linuron treatment (Cycoń et al. 2010). Bensulfuron-methyl had no influence on *E. adhaerens* SZMC 25856 and *P. resinovorans* (genotype I) SZMC 25872 in the range of 6.25-25 µg/ml, while it inhibited *P. resinovorans* (genotype II) SZMC 25875 at the highest tested concentration (25 µg/ml). Bensulfuron-methyl introduced in paddy soil samples negatively affected soil bacteria upon application, but this effect was relatively short in time, and the microbial communities recovered during the following 5 weeks (Lin et al. 2012). Furthermore, the pesticide was shown to have inhibitory effect on *B. velezensis* SZMC 6161J at 6.25, 12.5, and 25 µg/ml in a concentration-dependent manner (Vörös et al. 2019).

Among the 33 tested pesticides representing different chemical types and modes of action, some fungicides and herbicides suppressed the growth of *P. resinovorans* and *E. adhaerens* isolates, however, the strains tolerated the majority of the studied pesticides at levels that may occur in agricultural soils (6.25, 12.5, and 25 µg/ml, Vörös et al. 2019). No literature data are available about the influence of pesticides on *E. adhaerens* and *P. resinovorans*, therefore, their exploitable pesticide tolerance is reported here for the first time.

6.5.4 Salinity tolerance assay

The growth of *E. adhaerens* SZMC 25856 was significantly stimulated by 1.6 and 3.1 g/l ($p<0.05$), and significantly inhibited by 12.5 g/l NaCl ($p<0.05$) (Figure 19). *P. resinovorans* (genotype I) SZMC 25872 and (genotype II) SZMC 25875 grew sufficiently without significant inhibition ($p>0.05$) in the presence of 1.6-6.3 g/l NaCl and 1.6-12.5, respectively. Increasing the concentration to 25.0 g/l led to the complete growth inhibition of all 3 isolates (Figure 19).

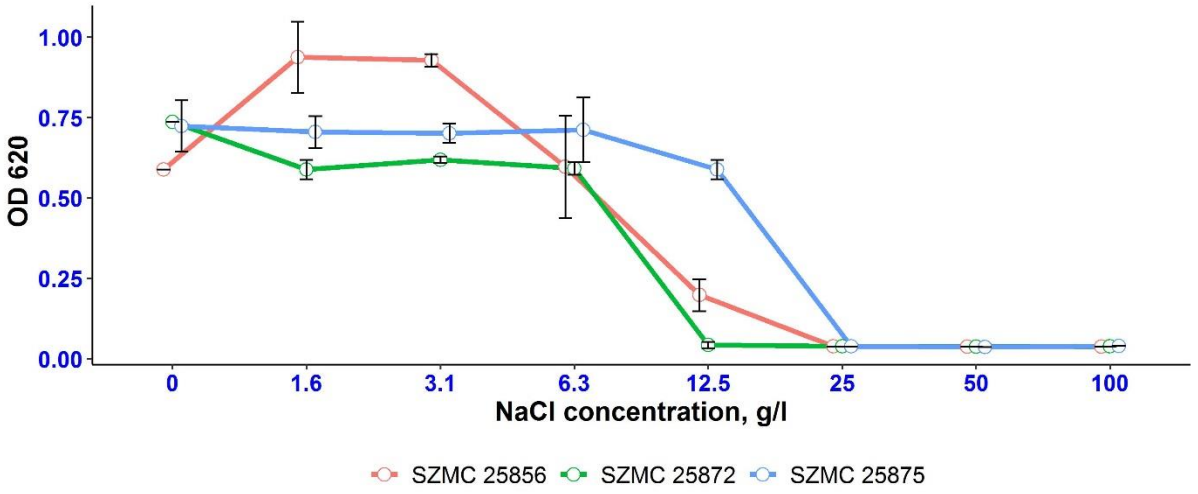

Figure 19. The effect of salinity (NaCl) on the growth of glyphosate-tolerant bacteria, *E. adhaerens* SZMC 25856, *P. resinovorans* (genotype I) SZMC 25872, and *P. resinovorans* (genotype II) SZMC 25875.

Soil salinization is a worldwide issue occurring in more than 100 countries. In 2015, the total area of salt-affected soils was estimated as 412 million hectares (FAO, Main Report 2015). The global area of soils impaired by salinity due to natural and human activities is growing by ten million hectares annually, which includes 1 million hectares within the European Union (Mokrani et al. 2020). All the examined *P. resinovorans* and *E. adhaerens* strains grew sufficiently in the presence of 1.6-6.3 g/l NaCl (Figure 19). *E. adhaerens* TMX-23 could not only to grow in the presence of 9 g/l NaCl, but also promoted the germination of soybean seeds (Zhou et al. 2013). Far higher salt tolerance (18-46 g/l) was reported by Alías-Villegas et al. (2015) for *E. meliloti* LMG 6133 and *E. medicae* LMG 19920. No data were found about the effect of salinity stress on *P. resinovorans*, but *P. aeruginosa* PDMZnCd2003 could resist 80 g/l NaCl (Nakbanpote et al. 2014). Salinity stress mostly appears to be species dependent. The optimal salinity level for the growth of *O. cytisi* IPA7.2 was found to be 10-20 g/l NaCl, while at 40 g/l its growth was inhibited by 50% (Burygin et al. 2019). Patel et al. (2017) reported that half of the 123 strains isolated from soil samples of natural hot springs could grow in the presence of 50 g/l NaCl, and *B. cereus* and *Aneurinibacillus aneurinilyticus* survived in the presence of 50 g/l NaCl.

As in our experiments, *E. adhaerens* SZMC 25856, as well as and *P. resinovorans* SZMC 25872 and SZMC 25875 tolerated 12.5 g/l NaCl. Therefore, they can be suggested to be able to grow in mildly saline environments, which considerably broaden their utilisation potential.

6.5.5 Drought tolerance test

E. adhaerens SZMC 25856 was able to grow sufficiently in the presence of 3.9-250 g/l PEG without significant inhibition ($p>0.05$), with the exception of 125 g/l, in which case the growth was significantly inhibited compared to the positive control ($p<0.05$) (Figure 20). *P. resinovorans* (genotype I) SZMC 25872 also grew in the presence of 3.9-250 g/l PEG, but the highest concentrations (125-250 g/l) significantly inhibited its growth ($p<0.05$). PEG applied at 3.9 and 31.3 g/l concentration significantly promoted the growth of *P. resinovorans* (genotype II) SZMC 25875, and this strain was significantly suppressed by PEG only at the highest tested concentration (250 g/l).

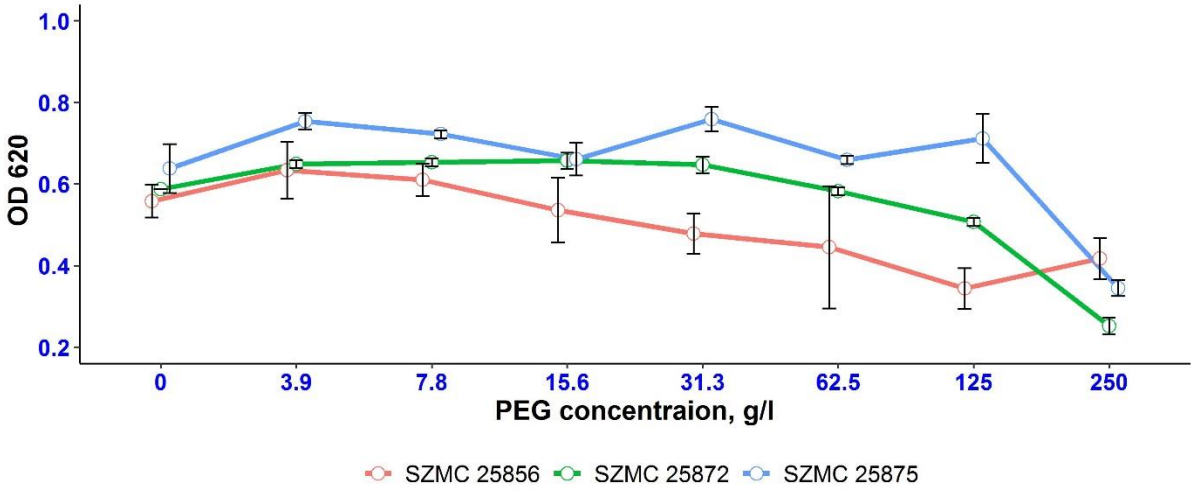

Figure 20. The effect of drought stress (PEG 6000) on the growth of glyphosate-tolerant bacteria, *E. adhaerens* SZMC 25856, *P. resinovorans* (genotype I) SZMC 25872, and *P. resinovorans* (genotype II) SZMC 25875.

As drought stress can also significantly inhibit plant growth and yield (Aslam et al. 2018, Devi et al. 2018), testing the drought tolerance of beneficial microorganisms is also an important step before their *in vivo* application. PEG is widely applied in various studies not only to evaluate the drought tolerance of bacteria (Wang et al. 2014, Kumari et al. 2016, Aslam et al. 2018, Devi et al. 2018), but also to examine plant growth under *in vitro* simulated drought stress conditions (Hamayun et al. 2010). In our studies, the examined *E. adhaerens* and *P. resinovorans* isolates could survive in the presence of up to 125 g/l PEG 6000 (Figure 20). Among the 150 tested bacterial strains, the growth was declined due to the increasing PEG concentration, and *B. marisflavi* WR2, *B. thuringiensis* WS11, and *B. subtilis* WL19 showed the highest drought tolerance (up to 400 g/l PEG) (Aslam et al. 2018). Devi et al. (2018) evaluated the growth of 44 bacterial strains under drought stress conditions and defined drought tolerance level as 300 g/l PEG. Most of the tested bacteria, 34 strains including *B. azotoformans* MTCC2953, *B. aryabhattai* KSBN2K7, and *Lactobacillus pentosus* M2T2B4 resisted this concentration. Nine bacterial isolates tested by Kumari et al. (2016) tolerated 50 g/l, while several strains, such as *P. simiae* AU, *P. koreensis* AK-1, and *Carnobacterium* sp. SJ-5 resisted even 150 g/l PEG.

As PEG at a 160 g/l concentration was considered as severe drought condition for soybean seedlings (Hamayun et al. 2010), and 300 g/l was the highest level for the selection of drought-tolerant bacterial strains in the studies by Devi et al. (2018), *E. adhaerens* SZMC 25856, as well as *P. resinovorans* SZMC 25872 and SZMC 25875 - being able to resist 125-250 g/l PEG - can also be considered to be drought-tolerant, which further enhances their potential *in vivo* applications.

6.6 Whole genome sequence analysis of selected glyphosate-tolerant bacteria

In agreement with the reference isolate (*E. adhaerens* Casida A), the whole genome sequence analysis has revealed two plasmids in addition to the chromosome in *E. adhaerens* SZMC 25856. According to our results, plasmid B of *E. adhaerens* SZMC 25856 contains considerable deletions compared to that of the reference strain. However, it is still likely to originate from the Casida A strain as the reads mapped to that plasmid B include the genes encoding for the replication proteins RepA, RepB, and RepC, which are present in plasmid A as well. For the analysis of the genome sequences of *P. resinovorans* (genotype I) SZMC 25872 and *P. resinovorans* (genotype II) SZMC 25875, *P. resinovorans* NBRC 106553 was selected as a reference. The reference genome consists of a chromosome and a plasmid (6,285,863 and 198,965 nt, respectively). The consensus plasmid length was very low in the case of both *P. resinovorans* strains (8619/198965 nt = 4.3 and 4562/198965 nt = 2.3 % for SZMC 25872 and SZMC 25875, respectively). Plasmid replication (*rep*) genes were not present in the consensus plasmid sequence, and thus it can be concluded that these short consensus sequences derive only from false positive mapping of some reads. In contrast with the reference isolate, the examined *P. resinovorans* strains lack the plasmid.

The analysis of the genome sequences has revealed the presence of various genes involved in the degradation of xenobiotics, the tolerance to heavy metals and antibiotics, QS, as well as the synthesis and tolerance to biogenic amines. Several of them were common in different isolates, while numerous genes (e. g. 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 examined strains, which might explain certain beneficial characteristics of them discovered during our studies (Subsections 6.5.2 and 6.5.3). Some relevant examples are listed in Table 23. In *E. adhaerens* SZMC 25856, a high number of such genes (such as alkanal monooxygenase, alkanesulfonate monooxygenase,

protocatechuate 3,4-dioxygenase, p-hydroxybenzoate hydroxylase, benzoate 1,2-dioxygenase, catechol 1,2-dioxygenase, 4-hydroxyphenylacetate 3-monooxygenase, heavy metal transporting ATPase) were found to be located on plasmid A suggesting its potential importance in xenobiotic and heavy metal tolerance.

Table 23. Examples of xenobiotic degradation (XD) and heavy metal tolerance (HMT)-related genes detected in glyphosate-tolerant bacteria (C: chromosome, A: plasmid A, B: plasmid B)

Protein	**Enzyme entry**	**Function**	**Location**
***E. adhaerens* SZMC 25856**			
Benzoate 1,2-dioxygenase	EC 1.14.12.10	XD	C, A, B
Quinone oxidoreductase	EC 1.6.5.5	XD	C
Alkanal monooxygenase alpha chain	EC 1.14.14.3	XD	A, B
Alkanesulfonate monooxygenase	EC 1.14.14.5	XD	A
Protocatechuate 3,4-dioxygenase	EC 1.13.11.3	XD	A
p-hydroxybenzoate hydroxylase	EC 1.14.13.2	XD	A
Catechol 1,2-dioxygenase	EC 1.13.11.1	XD	A
4-hydroxyphenylacetate 3-monooxygenase	EC 1.14.13.3	XD	A
Pb, Cd, Zn and Hg transporting ATPase	EC 3.6.3.3	HMT	A
***P. resinovorans* (genotype I) SZMC 25872**			
Quinone oxidoreductase	EC 1.6.5.5	XD	C
Phenol hydroxylase	EC 1.14.13.7	XD	C
Muconate cycloisomerase	EC 5.5.1.1	XD	C
Muconolactone isomerase	EC 5.3.3.4	XD	C
Catechol 1,2-dioxygenase	EC 1.13.11.1	XD	C
Benzoate 1,2-dioxygenase	EC 1.14.12.10	XD	C
5-carboxymethyl-2-hydroxymuconate delta-isomerase	EC 5.3.3.10	XD	C
3,4-dihydroxyphenylacetate 2,3-dioxygenase	EC 1.13.11.15	XD	C
Phenylacetaldehyde dehydrogenase	EC 1.2.1.39	XD	C
Cyclohexanone monooxygenase	EC 1.14.13.22	XD	C
Protocatechuate 3,4-dioxygenase	EC 1.13.11.3	XD	C
Aldehyde dehydrogenase	EC 1.2.1.3	XD	C
Phenylacetate degradation enoyl-CoA hydratase	EC 4.2.1.17	XD	C
Salicylate hydroxylase	EC 1.14.13.1	XD	C
Phosphonate ABC transporter	TC 3.A.1.9.1	HMT	C
Pb, Cd, Zn and Hg transporting ATPase	EC 3.6.3.3	HMT	C
Zn^{2+}-exporting ATPase	EC 3.6.3.5	HMT	C
Copper-translocating P-type ATPase	EC 3.6.3.4	HMT	C
***P. resinovorans* (genotype II) SZMC 25875**			
Quinone oxidoreductase	EC 1.6.5.5	XD	C
Alkanesulfonate monooxygenase	EC 1.14.14.5	XD	C
Phenol hydroxylase	EC 1.14.13.7	XD	C
5-carboxymethyl-2-hydroxymuconate delta-isomerase	EC 5.3.3.10	XD	C
3,4-dihydroxyphenylacetate 2,3-dioxygenase	EC 1.13.11.15	XD	C
Muconate cycloisomerase	EC 5.5.1.1	XD	C
Muconolactone isomerase	EC 5.3.3.4	XD	C

Catechol 1,2-dioxygenase	EC 1.13.11.1	XD	C
Benzoate 1,2-dioxygenase	EC 1.14.12.10	XD	C
Acetaldehyde dehydrogenase	EC 1.2.1.10	XD	C
Isoquinoline 1-oxidoreductase	EC 1.3.99.16	XD	C
p-hydroxybenzoate hydroxylase	EC 1.14.13.2	XD	C
Protocatechuate 3,4-dioxygenase	EC 1.13.11.3	XD	C
Aldehyde dehydrogenase	EC 1.2.1.3	XD	C
Phenylacetate degradation enoyl-CoA hydratase	EC 4.2.1.17	XD	C
Phosphonate ABC transporter	TC 3.A.1.9.1	HMT	C
Pb, Cd, Zn and Hg transporting ATPase	EC 3.6.3.3	HMT	C
Zn^{2+}-exporting ATPase	EC 3.6.3.5	HMT	C
Copper-translocating P-type ATPase	EC 3.6.3.4	HMT	C

Enzymes identified in the genome of glyphosate-tolerant bacteria act in different cellular pathways of the xenobiotic degradation and tolerance to heavy metals and pesticides. The degradation of phenol catalyzed by phenol hydroxylase was reported in *B. thermoglucosidasius* A7 (Kirchner et al. 2003). Catechol 1,2-dioxygenases were reported to play role in the remediation of wastewater polluted with the herbicide diuron and other xenobiotics (Setlhare et al. 2018). Alkanesulfonate monooxygenase system found in *E. adhaerens* SZMC 25856 is applied by various bacteria to acquire sulfur from sulfur-containing compounds at sulfur-limited environement (Ellis 2011). The presence of several heavy metal transporting ATPase, such as Pb-Cd-Zn-Hg transporting ATPase identifed in all the 3 tested strains might play remarkable role in the efflux of metals from the cytoplasm (Argüello et al. 2011). In conclusion, these molecular insights have revealed several possible systems applied by glyphosate-tolerant bacteria to overcome abiotic stress factors, particularly the presence of various heavy metals and pesticides.

7 CONCLUSIONS AND FUTURE PERSPECTIVES

In the present study, glyphosate-tolerant bacterial strains were isolated and examined. Two strains – *E. adhaerens* SZMC 25856 and *P. resinovorans* (genotype II) SZMC 25875 were found to stimulate tomato growth significantly, while *P. resinovorans* (genotype I) SZMC 25872 was revealed as a potential biocontrol agent to suppress the growth of the plant pathogen *A. tumefaciens*. These strains were further examined for their plant growth-promoting traits, such as siderophore synthesis and ammonia production, which could provide crops with available iron and nitrogen source, respectively. Furthermore, *E. adhaerens* SZMC 25856 and *P. resinovorans* (genotype I) SZMC 25872 were found to synthesize IAA, an essential phytohormone for plant growth, which remarkably enhances their potential for agricultural utilisation. Both the living culture and the CCF of *P. resinovorans* (genotype I) SZMC 25872 could suppress *A. tumefaciens*. The highest growth inhibition was observed upon the application of succinic acid or L-alanine was as the sole carbon source. Based on our findings, siderophore-mediated suppression may be suggested as a potential mode of action in the case of CCF obtained from succinic acid-containing medium, while the inhibitory effect of CCF from the medium supplemented with L-alanine might be attributed to the observed trypsin and chymotrypsin-like protease activities, as well as the potential interference with the QS of *A. tumefaciens*. The HPLC-HRMS analysis of the CCF samples revealed the presence of several potential active metabolites, which might also play role in the inhibition of plant pathogens.

As adverse abiotic factors, such as pesticides and heavy metals present in agricultural soils, or salt and drought stress appear during the vegetation period and might remarkably limit the activity of the introduced beneficial microorganisms, the comprehensive eco-physiological characterisation of the potential biofertilisers and biocontrol strains is an essential prerequisite for their field application. In our studies, all the three selected strains could tolerate the presence of a variety of pesticides and heavy metals without significant inhibition. Moreover, these isolates possessed suitable tolerance to salt and drought stress conditions.

Altogether, the plant growth-promoting characteristics, biocontrol potential, and stress tolerance of the isolated glyphosate-tolerant strains make them promising candidates for utilisation in various agricultural applications, even under unfavourable abiotic conditions. The subsequent studies might include the examination of these strains in greenhouse and under *in situ* conditions. Furthermore, the examination of the effect of glyphosate-tolerant strains on tomato growth under abiotic stress factors including the presence of glyphosate and heavy metals will be of high practical importance. Furher investigations are required to verify the biocontrol potential of the detected metabolites produced by both *P. resinovorans* SZMC 25872 and SZMC 25875 in medium supplied with L-alanine or succinic acid as carbon sources. Studies of the strains in biotechnological applications or in bioremediation of polluted substrates might also expand their applied potential.

8 SUMMARY

Soils represent the fundamental basement of agriculture, but human pressure is reaching their critical limits, and a meaningful soil management is crucial to preserve the ecosystems and biodiversity (FAO, Main Report, 2015). The extensive use of synthetic pesticides and fertilisers has resulted in soil and water contamination, rising subsequent risks for the environment and human health as well. Pesticides, including the most widely-used herbicide glyphosate, their residues, and heavy metals have deleterious effect on all living organisms, including beneficial soil microorganisms, bees, fish, and mammals (Gouda et al. 2018, Rahman et al. 2018, Van Bruggen et al. 2018). Therefore, sustainable agricultural strategies involving environment-friendly practices are of a great interest to enhance crop productivity and to maintain soil fertility. Fundamental processes in soil systems, such as nutrient cycling, carbon storage, and the maintenance of soil fertility are regulated by soil microorganisms (Aislabie and Deslippe 2013). Furthermore, a variety of soil microorganisms cannot only promote crop growth, but also protect the host plants from various plant pathogens and stimulate the tolerance of plants to adverse abiotic stress factors. Beneficial strains can enhance plant growth and protect crops via direct and indirect mechanisms, including ammonia and siderophore production, the synthesis of phytohormones, the solubilisation of phosphorus and potassium, and the secretion of bioactive metabolites. Therefore, microbe-based biofertilisers and biocontrol strains are effective and environment-friendly alternatives to hazardous chemical pesticides (Gouda et al. 2018, Basu et al. 2021). However, the successful application of microbial strains with the potential of plant growth stimulation and crop protection needs to be well-considered, and requires the careful eco-physiological characterisation of the beneficial strains, especially the examination of their stress tolerance, as certain abiotic factors can remarkably limit their activity *in vivo* (Basu et al. 2021).

In our studies, 30 bacterial strains were isolated from glyphosate-treated soil, and their growth was examined in the presence of 1 g/l glyphosate as the sole carbon and nitrogen source. The sequence analysis of fragments of the 16S rRNA and the RNA polymerase subunit beta (*rpoB*) gene performed in the case of 10 selected strains showing the most intensive growth has revealed that 2 strains belong to the species *Ensifer adhaerens*. The remaining seven isolates were identified as *Pseudomonas resinovorans*, representing two different genotypes (I and II, involving 3 and 4 isolates, respectively). A single strain was classified as *Ochrobactrum anthropi*, but as this species is known as opportunistic human pathogen, it was excluded from the subsequent studies. The previous metagenome analysis also detected higher abundance of both *E. adhaerens* and *P. resinovorans* in the glyphosate-treated soil compared to the control sample.

A significant increase in the root length of tomato seedlings was observed upon the treatment by *E. adhaerens* SZMC 25856 and *P. resinovorans* (genotype II) SZMC 25875 compared to water and potato dextrose broth (PDB) controls and the remaining 7 isolates $p<0.05$). Both the living culture and the cell-free culture filtrates of *P. resinovorans* (genotype I) SZMC 25872 could significantly inhibit the growth of the plant pathogen *Agrobacterium tumefaciens*. Therefore, the mentioned three glyphosate-tolerant strains were selected for the detailed examination of their plant growth-promoting potential, the mechanisms involved in pathogen suppression, and the eco-physiological characterisation.

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) in PDB with and without tryptophan, respectively. All the three strains: *E. adhaerens* SZMC 25856, *P. resinovorans* (genotype I) SZMC 25872 and *P. resinovorans* (genotype II) SZMC 25875 could synthesize siderophores (21.66, 24.59 and 13.73%, respectively) in the presence of succinate as the

carbon source. The effect of carbon source was crucial for the siderophore production: the highest siderophore amount of all strains was detected in Minimal Medium (MM) supplied with L-alanine or succinic acid as sole carbon source, while no siderophores were detected in the remaining media, such as Luria-Bertani (LB), yeast extract glucose (YEG), PDB, and MM amended with glucose. All isolates, regardless of species and genotypes, were found to produce remarkably high amount of ammonia (0.11-0.14 mg/ml), while none of them was found to posses phosphate-solubilising activity.

Among the *P. resinovorans* strains, SZMC 25872 (genotype I) showed the most remarkable inhibition of *A. tumefaciens* SZMC 14557 in dual-culture assays. The carbon source utilisation tests performed with the two strains have revealed that 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) can be utilised by both isolates as the sole carbon source. The effect of these substances on the biocontrol efficacy of *P. resinovorans* SZMC 25872 was evaluated, and the strongest suppression of *A. tumefaciens* was observed in the case of sodium pyruvate, L-alanine, succinic acid, and D-glucose. The cell-free culture filtrates (CCF) obtained from the supernatant of *P. resinovorans* SZMC 25872 grown in 7 different media, particularly MM amended with L-alanine and succinic acid, significantly inhibited the growth of *A. tumefaciens* at 25-50% concentrations. Regarding the inhibitory activity of *P. resinovorans* SZMC 25872 in the presence of succinic acid, siderophore-mediated suppression of *A. tumefaciens* can be suggested, as the inhibitory effect caused by these CCF samples, which was eliminated by the addition of extra iron. However, no difference in the inhibition was observed upon treatments with CCF obtained from L-alanine-containing medium regardless of additional iron. This finding suggests different modes of inhibition in the case of CCFs obtained from the culture supernatants of *P. resinovorans* SZMC 25872 grown in the presence of succinic acid and L-alanine. The HPLC-HRMS analysis has revealed a total of 24 previously unknown metabolites with the inhibitory potential in L-alanine and succinic acid containing media (12-12 compounds in each). 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 only in these 2 media at substantial quantities compared to the remaining 5 tested media, containing no or just negligible traces of these metabolites. Based on the identical molecular weight and fragmentation pattern, 9 compounds produced 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 nine compounds were found to have similar composition to homoserine lactones, but their main fragment differs from that of lactones reported in the literature. These compounds might have similar thermal stability as lactonase, because the growth of *A. tumefaciens* was stronger in the presence of CCF heated at 90 ºC for 30 min compared to untreated sample. Temperature affected only the compounds identified in the CCF from succinic acid-containing MM, while the inhibition caused by the CCF from MM amended with L-alanine, with no metabolites similar to lactone detected, was the same between heated and non-heated samples. The remaining 3 compounds discovered in succinic acid-containing medium might act as siderophores because their quantity was higher than in the remaining media. The inhibition effect caused by CCF from this medium was eliminated in the presence of an additional iron source. The metabolites detected in L-alanine-MM represent various fragmentation patterns, retention time, and molecular weight, which were not found in the current literature and available databases. As *P. resinovorans* has not been reported as a potential biocontrol strain yet, furhter examinations of these novel active metabolites is required to identify specific inhibitory compounds and their properties. Furthermore, enzymes such as extracellular trypsin and chymotrypsin-like protease were

detected in CCFs containing sodium pyruvate and particularly L-alanine as carbon source, suggesting the potential involvement of these enzymes in the inhibition of *A. tumefaciens*.

The comprehensive eco-physiological characterisation of the three selected glyphosate-tolerant strains revealed their substantial tolerance to various adverse abiotic stress factors. The optimal pH range for all isolates was found to be 6.59-8.95. 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. Moreover, 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 three strains. Salinity up to 6.3 g/l NaCl did not limit the growth of the tested glyphosate-tolerant bacteria either, while all strains could grow without significant inhibition up to 125 g/l polyethylene glycol (PEG) concentration, simulating drought stress, which enhances their potential of application in saline soils and under drought conditions.

The above-mentioned tolerance to abiotic stress factors, including xenobiotic and heavy metal tolerance was supported by the results of the whole genome 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.

E. adhaerens SZMC 25856 and *P. resinovorans* (genotype II) SZMC 25875 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* (genotype I) SZMC 25872 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.

9 ÖSSZEFOGLALÓ

A mezőgazdaságban a talajok alapvető jelentőséggel bírnak, azonban az emberiség kezdi elérni a földművelés során történő kihasználhatóságuk határait. A felelős talajkezelés létfontosságú az ökoszisztémák és a biodiverzitás megóvása szempontjából (FAO, Main Report, 2015). A szintetikus növényvédőszerek és műtrágyák széleskörű használata a talaj és a vizek szennyeződéséhez vezetett, mely veszélyt jelent mind a környezetre, mind pedig az emberek egészségére. A növényvédőszerek - köztük a leggyakrabban használt gyomirtó, a glifozát - és bomlástermékeik, valamint a nehézfémek ártalmasak minden élőlényre, így a hasznos talajbaktériumokra, méhekre, halakra és emlősökre is (Rahman és mtsai. 2018, Gouda és mtsai. 2018, Van Bruggen és mtsai. 2018). Az előbbiek miatt a fentartható mezőgazdasági technológiák, beleértve a környezetbarát módszereket, nagy jelentőséggel bírnak a terméshozam és a talaj termékenységének növelésében. Mikroorganizmusok szabályozzák a talajban az olyan alapvető folyamatokat, mint a tápanyagok körforgása, a szénraktározás, valamint a termékenység fenntartása (Aislabie és Deslippe 2013). Továbbá egyes mikroorganizmusok a növények növekedésének elősegítésén túl védelmet biztosítanak különféle kórokozók ellen, valamint abiotikus stresszfaktorokkal szemben is fokozzák azok ellenállóképességét. A kedvező hatású mikróbatörzsek képesek elősegíteni a növények növekedését és védelmet is nyújtanak, mely történhet direkt és indirekt mechanizmusokon keresztül, mint az ammónia, sziderofórok és növényi hormonok termelése, foszfor- és káliumforrások mobilizálása, valamint bioaktív metabolitok kiválasztása. Emiatt a mikroorganizmusokon alapuló biotrágyák és biokontroll törzsek hatékony és környezetbarát alternatívának tekinthetők a veszélyt jelentő kémiai növényvédőszerekkel szemben (Gouda és mtsai. 2018, Basu és mtsai. 2021). A növények növekedésének serkentésére és azok védelmére potenciálisan felhasználható mikróbatörzsek alkalmazása azonban alapos körültekintést igényel, szükséges a törzsek alapos ökofiziológiai jellemzése, különös tekintettel a stressztűrő képességükre, mivel egyes abiotikus tényezők jelentősen ronthatják a törzsek hatékonyságát *in vivo* (Basu és mtsai. 2021).

Munkánk során 30 törzset izoláltunk glifozáttal kezelt talajból, melyek növekedését megvizsgáltuk 1 g/l glifozát, mint egyedüli szén- és nitrogénforrás jelenlétében. A tíz leggyorsabban növekvő törzset a 16S rRNS gén az RNS polimeráz béta alegységének (*rpoB*) szekvenciái alapján azonosítottuk. Eredményeink szerint két törzs az *Ensifer adhaerens*, hét a *Pseudomonas resinovorans*, egy pedig az *Ochrobactrum anthropi* fajhoz tartozik. Ez utóbbi opportunista humán patogénként ismert, ezért kizártuk a további vizsgálatokból. A *Pseudomonas resinovorans* izolátumok két elkülönülő genotípust képviselnek (I: 3, II: 4 törzs). Az előzetesen elvégzett metagenomikai vizsgálat során kimutattuk, hogy az *E. adhaerens* és a *P. resinovorans* fajok nagyobb mennyiségben vannak jelen a glifozáttal kezelt talajmintában a kontrollhoz képest.

Az *E. adhaerens* SZMC 25856 és *P. resinovorans* SZMC 25875 (II-es genotípus) törzsekkel kezelt paradicsompalánták esetén szignifikáns növekedést tapasztaltunk a gyökerek hosszában a vízzel és PDB-vel (burgonyadextróz tápoldat) kezelt kontrollok, valamint a másik hét törzzsel kezelt növényekhez képest ($p<0,05$). A *P. resinovorans* SZMC 25872 (I-es genotípus) esetén megfigyeltük, hogy mind az élő tenyészet, mind pedig annak sejtmentes fermentleve képes volt szignifikáns gátló hatást kifejteni az *Agrobacterium tumefaciens* növekedésére. Ezt a három glifozátrezisztens törzset választottuk ki a növényi növekedés serkentése és a kórokozók gátlása hátterében álló mechanizmusok, valamint az ökofiziológiai jellemzőik részletes vizsgálatára.

Az *E. adhaerens* SZMC 25856 és *P. resinovorans* SZMC 25872 (I-es genotípus) törzsek esetén kimutattuk, hogy 0.28-0.23 illetve 0.004-0.02 µg/ml indol-3-ecetsavat termelnek PDB tápoldatban triptofán hozzáadása mellett és anélkül is. Mindhárom törzs

esetén megfigyeltük, hogy képesek szideróforok termelésére borostyánkősav szénforrás jelenlétében: az *E. adhaerens* SZMC 25856 esetén 21,66, a *P. resinovorans* SZMC 25872 (I-es genotípus) esetén 24.59, míg *P. resinovorans* SZMC 25875 (II-genotípus) esetén 13,73%-os szideroफórtermelés volt megfigyelhető. A különböző szénforrásoknak meghatározó szerepük volt a szideroफórtermelés szabályozásában. A legintenzívebb szideroफórtermelést mindegyik törzs esetében minimál tápoldatban figyeltük meg, melyben L-alanin vagy borostyánkősav jelentette az egyedüli szénforrást, míg LB (Luria-Bertani), PDB, YEG, (élesztőkivonat-glükóz) és glükózzal kiegészített minimál tápoldat alkalmazása esetén nem mutattunk ki szideroफórokat. Továbbá mindhárom törzs esetén, fajtól és genotípustól függetlenül, jelentős ammóniatermelést tapasztaltunk (0,14-0,16 mg/ml), azonban foszfátmobilizáló aktivitást egyik törzs esetében sem sikerült kimutatni.

Az antagonizmus tesztek során a *P. resinovorans* SZMC 25872 izolátum (I-es genotípus) bizonyult a leghatékonyabbnak az *A. tumefaciens* SZMC 14557 törzs gátlásában. A két törzs által hasznosítani képes szénforrások azonosítására szintén végeztünk teszteket, melyek eredményeképp találtunk tizenhárom olyan vegyületet, melyeket mindkét törzs képes egyedüli szénforrásként hasznosítani. Ezek a 2-keto-D-glükonsav, D-fruktóz, D-glükuronsav, D-glükóz, glicerin-l-monoacetát, L-alanin, L-aszparagin, L-glutaminsav, L-glutamin, L-almasav, nátrium-piruvát, keményítő, és borostyánkősav voltak. Ezen vegyületeknek a *P. resinovorans* SZMC 25872 biokontroll képességének hatékonyságára gyakorolt hatását is vizsgáltuk, melynek eredményékép azt kaptuk, hogy az *A. tumefaciens* törzs a nátrium-piruvát, L-alanin, borostyánkősav, és D-glükóz esetén szenvedte el a legnagyobb gátlást. A *P. resinovorans* SZMC 25872 tenyészet felülúszójából nyert sejtmentes fermentlé mind a hét szénforrás esetén szignifikáns gátló hatást fejtett ki 25-50%-os koncentrációban az *A. tumefaciens* törzsre, különösen azok a minimál tápoldatok, melyekhez L-alanint és borostyánkősavat adtunk szénforrásként. Valószínűsíthetjük, hogy a *P. resinovorans* SZMC 25872 izolátum borostyánkősavat tartalmazó tápoldatból származó sejtmentes fermentleve által az *A. tumefaciens* törzsnövekedésére kifejtett gátló hatás a szideroफórok termelésén keresztül valósul meg, mivel a tápoldat vasforrással történő kiegészítése hatására a gátlás megszűnik, azonban a szénforrásként L-alaninnal kiegészített tápoldat esetén ez a jelenség nem figyelhető meg. Ezekből az eredményekből arra következtethetünk, hogy a *P. resinovorans* SZMC 25872 törzs sejtmentes fermentleve különböző hatásmechanizmusokon keresztül fejti ki hatását, ha a tápoldat borostyánkősav illetve L-alanin szénforrást tartalmaz. Az L-alanin- és borostyánkősav-tartalmú sejtmentes fermentléből 12-12, azaz összesen 24 eddig ismeretlen anyagcsereterméket sikerült azonosítanunk HPLC-HRMS (nagy teljesítményű folyadékkromatográfia-nagy felbontású tömegspektroszkópia) vizsgálatok segítségével, melyek szerepet játszhatnak az antagonizmus megvalósításában. Valószínűsíthetjük, hogy az SZMC 25872 és 25875 törzsek esetében nyert sejtmentes fermentlé gátló hatása ezeknek a még ismeretlen anyagcseretermékeknek köszönhető, mivel ezek a vegyületek csak az említett két szénforrás esetében voltak jelentős mennyiségben kimutathatók, szemben a másik öttel, ahol csak elhanyagolható mennyiségben vagy egyáltalán nem voltak jelen. Az azonos molekulatömegre és fragmentációs mintázatra alapozva a borostyánkősavat tartalmazó fermentlé esetében kilenc vegyületet három kémiai csoportra tudtuk osztani, négy, három, illetve két vegyülettel az egyes csoportokban. Továbbá mind a kilenc vegyületről sikerült megállapítanunk, hogy összetételük megegyezik a homoszerin laktonokéval, azonban fő fragmentumuk nem azonos a laktonok szakirodalomban leírt fő fragmentumával. Az *A. tumefaciens* jobban nőtt azokban az esetekben, amikor a fermentlevet, amivel a gátlást vizsgáltuk, 90 °C-os hőkezelésnek tettük ki 30 percig, a hőkezelésen át nem esett kontrollal történt kezeléshez képest, amiből arra következtettünk, hogy ezeknek a vegyületeknek a hőstabilitása megegyezik a laktonázéval. A hőkezelés csak a borostyánkősav-tartalmú fermentléből kimutatott vegyületekre volt hatással,

az L-alanint tartalmazó fermentléből nem mutattunk ki laktonszerű vegyületeket és a hőkezelt minta növekedést gátló hatása sem csökkent a nem hőkezelt kontrollhoz képest. Valószínűnek tartjuk, hogy a borostyánkősav-tartalmú fermentlében kimutatott maradék három vegyület szideroforként viselkedik, ezek mennyisége nagyobb volt, mint a többi tápoldat esetén, illetve a gátló hatás vasforrás hozzáadásával megszüntethető volt. Az L-alanin tartalmú fermentléből kimutatott vegyületek különböző fragmentációs mintázattal, retenciós idővel és molekulatömeggel rendelkeztek, sem a szakirodalomban, sem pedig az elérhető adatbázisokban nem találtuk meg őket. Mivel a *P. resinovorans* biológiai védekezésben történő felhasználásáról még nincs adat a szakirodalomban, a kimutatott új anyagcseretermékek további vizsgálata szükséges a gátlásért felelős vegyületek azonosításához és tulajdonságaik meghatározásához. A szénforrásként nátrium-piruvátot és különösen az L-alanint tartalmazó sejtmentes fermentlében sikerült kimutatnunk különböző extracelluláris enzimek, mint a tripszin és kimotripszin-szerű proteázok aktivitását, ami arra enged következtetni, hogy ezeknek az enzimeknek szerepük lehet az *A. tumefaciens* növekedésének gátlásában.

A három glifozáttoleráns törzzsel elvégzett széleskörű ökofiziológiai vizsgálatsorozat eredményei alapján megállapíthatjuk, hogy bizonyos abiotikus stresszfaktorokkal szemben nagymértékben ellenálónak bizonyultak. Mindegyik törzs esetén azt találtuk, hogy pH optimumuk 6,09-8,95 közé esett. Valamennyi törzs képes volt növekedni a vizsgált nehézfémek jelenlétében, 0,1 mM-os koncentrációban, mint a vas, a mangán, az ólom, és a réz. A *P. resinovorans* SZMC 25872 (I-es genotípus) 1 mM-os koncentrációig képes volt tolerálni a vasat és az ólmot, míg az *E. adhaerens* SZMC 25856 törzs képes volt növekedni 1 mM-os vas-, mangán- és ólom-koncentráció esetében is. A *P. resinovorans* SZMC 25875 törzs az előbbiek mellett pedig képes volt növekedni 1 mM-os koncentrációjú cink és alumínium jelenlétében is. Továbbá a tesztelt 33 növényvédőszer többségének, beleértve olyan gyomirtókat, mint a glifozát, benzulfuron-metil, klorotoluron, klórprofám, cinoszulfuron diuron, fenuron, izoproturon, primiszulfuron-metil, profám, 2,4-D (2,4-diklórfenoxiecetsav); illetve a gombaölőszereket, mint a karbendazim, karboxin, fenarimol, flutriafol, imazalil, tiabendazol, és tiofanát-metil és a rovarölőszert a diflubenzuront, nem volt káros hatása a három törzs egyikére sem. Megállapítottuk, hogy a sóstressz nem gátolta a vizsgált glifozát toleráns baktériumok növekedését 6,3 g/l NaCl sókoncentrációig, valamint mindegyik törzs képes volt növekedni szignifikáns gátló hatás nélkül 125 g/l PEG (polietilén-glikol) koncentrációig, ami jól szimulálja a szárazságstresszt, így feltételezhetjük, hogy alkalmazhatóak lehetnek magasabb sótartalmú talajban, illetve száraz körülmények között is.

Az izolátumok különféle abiotikus stresszfaktorokkal (xenobiotikumok, nehézfémek) szembeni ellenálló képességét teljes genomszekvencia-vizsgálattal is sikerült alátámasztanunk. Sikerült kimutatnunk olyan gének jelenlétét, melyek szerepet játszanak a xenobiotikumok lebontásában, a nehézfémekkel és antibiotikumokkal szembeni toleranciában, quorum sensing-ben, biogén aminok termelésében és a velük szembeni toleranciában. Ezek közé tartozik a benzoesav-1,2-dioxigenáz, protokatechusav-3,4-dioxigenáz, kinon-oxidoreduktáz, katekol-1,2-dioxigenázt, valamint az ólom-, kadmium-, cink- és higanytranszportáló ATP-ázt.

Az *E. adhaerens* SZMC 25856 és *P. resinovorans* SZMC 25875 (II-es genotípus) törzsekről megállapítottuk, hogy képesek szignifikánsan serkenteni a paradicsomnövények növekedését, valamint rendelkeznek olyan növénynövekedést elősegítő képességekkel, mint az indol-3-ecetsav szintézise, illetve szideroför- és ammóniatermelés. A *P. resinovorans* SZMC 25872 (I-es genotípus) törzs az előbbiekhez hasonlóan szintén képes serkenteni a növények növekedését, továbbá képes szignifikáns gátló hatást gyakorolni az *A. tumefaciens* növekedésére mind élő tenyészetként, mind pedig a termelt szideroforok, proteázok és még ismeretlen bioaktív anyagcseretermékek segítségével. Mindhárom törzs jelentős toleranciát

mutatott különböző abiotikus stresszfaktorokkal szemben, köszönhetően a különböző stresszfaktorokkal szembeni rezisztenciát biztosító gének jelenlétének, ezáltal alátámasztva a sikeres mezőgazdasági alkalmazás lehetőségét.

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