UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY
ACADEMIC STUDY IN MICROBIOLOGY

Domen TRAMPUŽ

GENETIC DIVERSITY OF *Bacillus mycoides* ISOLATED FROM THE TUBERS OF THE POTATO PLANT, ITS RHIZOSPHERE AND THE BULK SOIL

M. SC. THESIS

Master Study Programmes - 2. Field Microbiology

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GENETSKA RAZNOLIKOST BAKTERIJ VRSTE *Bacillus mycoides* IZOLIRANIH IZ TAL, RIZOSFERE IN GOMOLJEV KROMPIRJA

MAGISTRSKO DELO
Magistrski študij – 2. stopnja Mikrobiologija

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The M.Sc. Thesis is a completion of the Master Study Programme in Microbiology. The work was carried out in the laboratory of the Microbial Ecology Department, Faculty of Mathematics and Natural Sciences, University of Groningen, Groningen, The Netherlands, and in the laboratory of the Chair of Microbiology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia.

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AB *Bacillus mycoides* is a Gram positive, soil dwelling bacterium that is taxonomically a part of *Bacillus cereus* sensu lato clade and has a unique and recognisable morphology. The bacterium, although not previously described as potato endophyte, has been recently implicated as a potential potato endophyte. To better understand its endophytic interactions we analysed the diversity and structure of *B. mycoides* populations in endosphere, rhizosphere and the surrounding soil of potato fields. We isolated 350 isolates from two cultivars of potato; Miranda and Seresta, and sampled the soil of garden beds in which they were planted. For the analysis of the diversity and structure of *B. mycoides* populations in these environments, we used ERIC-PCR, a genomic fingerprinting technique based on multiple non-coding sequences, which are dispersed throughout the bacterial genome and are common to many species of bacteria. We analysed 146 isolates from cultivar Miranda and bulk soil and found that they clustered into 24 ERIC types. The results of diversity estimation showed highest diversity index for the bulk soil population, lower diversity for the rhizosphere population and the lowest diversity index for the endosphere population. We found that on average 50% of the endosphere isolates belonged to the same ERIC type. We also analysed the diversity and distribution of PlcR-PapR quorum sensing systems by partial sequencing of 101 genes of randomly selected isolates from bulk soil, rhizosphere and endosphere. The results revealed that the analysed strains cluster in two out of four known clusters, which represent communication specificity groups or pherotypes. The genes within these groups were highly similar and the distribution of individual pherotypes amongst endosphere and rhizosphere of all plants was equal, whereas the distribution amongst rhizosphere and endosphere of a single plant was unequal, with two plants having a different dominant pherotype in rhizosphere or endosphere and a third plant having the same dominant pherotype in endosphere and rhizosphere.

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AI *Bacillus mycoides* je Gram pozitivna bakterija, ki jo najdemo predvsem v zemlji in sodi v taksonomsko skupino *Bacillus cereus* sensu lato (v širšem smislu). Zanjo je značilna unikatna in lahko prepoznavna morfologija. Bakterija do nedavnega ni bila prepoznana kot endofit krompirja, vendar so pred kratkim ugotovili, da bi lahko potencialno le-to lahko bila. Za boljše razumevanje omenjene interakcije smo raziskovali raznolikost in strukturo populacije vrste *B. mycoides* v endosferi, rizosferi in v zemlji krompirjevega polja, od koder smo pridobili vzorce. Skupno smo izolirali 350 bakterij vrste *B. mycoides* in sicer iz dveh kultivarov rastlin (Miranda in Seresta) in iz zemlje obeh poljskih gred. Za analizo raznolikosti in strukture populacij *B. mycoides* v tem okolju, smo uporabili tehniko ERIC-PCR. Le-ta temelji na pomnoževanju več nekodirajočih zaporedij, razpršenih po celotnem bakterijskem genomu, ki so značilna za več različnih vrst bakterij in jo uvrščamo med tehnike pridobivanja genetskega prstnega odtisa. Analizirali smo 146 izolatov pridobljenih iz kultivarja Miranda in iz zemlje obeh gred (Miranda in Seresta), ter odkrili 24 operacijskih taksonomskih enot, ki smo jih poimenovali ERIC tipi. Rezultati ocen raznolikosti so pokazali najvišjo raznolikost znotraj populacij *B. mycoides* v zemlji obeh gred. Nižje je bila ocenjena raznolikost populacij v rizosferi, najnižja ocena raznolikosti pa je blila izračunana za populacije v endosferi. Ugotovili smo tudi, da v povprečju 50 % izolatov iz endosfere vsake rastline posebej sodi v en ERIC tip. Analizirali smo tudi raznolikost in porazdelitev sistemov zaznavanja kvoruma PlcR-PapR. Analizo smo izvedli s sekvenciranjem 101 naključno izbranega izolata iz zemlje gred, rizosfere in endosfere. Rezultati so pokazali, da se analizirani sevi razvrstijo v dve izmed štirih znanih gruč, ki predstavljajo specifične komunikacijske skupine oz. ferotipe. Geni znotraj teh skupin so bili izrazito podobni, porazelitev posameznega ferotipa med endosfere in rizosfere vseh rastlin pa je bila enakovredna. Porazdelitev ferotipov med rizosfero in endosfero posameznih rastlin pa je bila različna, pri čemer sta imeli dve rastlini različne prevladujoče ferotipe v rizosferi ali endosferi in pri čemer je imela tretja rastlina isti ferotip prevladujoč tako v rizosferi kakor tudi v endosferi.

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ABBREVIATIONS AND SYMBOLS

DGGE	Denaturing gradient gel electrophoresis
dH_2O	Deionised water
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates mix
EDTA	Ethylenediaminetetraacetic acid
ERIC-PCR	Enterobacterial repetitive intergenic consensus PCR technique
HCl	Hydrochloric acid
$MgCl_2$	Magnesium chloride buffer
MLEE	Multi locus enzyme electrophoresis
MLST	Multilocus sequence typing
NaCl	Sodium chloride
OTU	Operational taxonomic unit.
PBS	Phosphate buffered saline solution
PCR	Polymerase chain reaction technique
PFGE	Pulse field gel electrophoresis
PlcR-1, PlcR-2, PlcR-3, PlcR-4	An unofficial short name for pherotypes of PlcR receptor (PlcR group I, II, III and IV respectively).
PlcR-PapR	Quorum sensing system of *B. cereus* taxonomic group
plcR-papR	Locus coding the PlcR-PapR quorum sensing system
rep-PCR	Repetitive Sequence-based PCR technique
s. l.	sensu lato (in broader sense)
s. s.	sensu stricto (in stricter sense)
SDS	Sodium dodecyl sulphate
TES	Tris, EDTA, Sodium chloride buffer

1 INTRODUCTION

Potato (*Solanum tuberosum*) is a plant that is susceptible to many different diseases, some of which develop due to pathogenic bacteria. Upon microbiologically inspecting the tubers with observed glassy discoloration on the inside of the potato, colonies with fungal-like morphology were observed which morphologically resembled *Bacillus mycoides* (Jan Spoelder et al., unpublished data). To confirm that, a piece of tuber was inoculated on a nutrient agar plate resulting in growth of fungal-like colonies. Based on their 16S rDNA gene sequence and their morphology, the colonies were identified as *Bacillus mycoides*. *Bacillus mycoides* is taxonomically a part of *Bacillus cereus* sensu lato clade along with *B. thuringiensis*, *B. anthracis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides* and *B. cytotoxicus*. This bacterium has not yet been intensively studied, especially in regards of its interactions with plants. Similarly, *B. mycoides* has not been previously described as a potato endophyte. In this work, we aimed to better our understanding of plant - bacterial interactions by performing the analyses of the diversity and structure of *B. mycoides* populations in endosphere, rhizosphere and the surrounding soil of potato fields. To this aim, we isolated *B. mycoides* from two cultivars of potato and from the soil of garden beds in which they were planted. To evaluate the diversity of this bacterium used the ERIC-PCR technique, which is a genomic fingerprinting technique, based on the PCR amplification of the non-coding sequences that scattered throughout the genome of bacterial cells, and are common to many species of bacteria (Versalovic et al., 1991; Versalovic et al., 1994). We also addressed their diversity by calculating Shannon-Wiener diversity indices.

Recently, quorum sensing compounds emerged as important modulators of microbe-plant interactions (Hartmann et al., 2014). Bacteria use quorum sensing systems to communicate (Miller and Bassler, 2001). Usually there are several different “languages” or versions of the same quorum system, which utilise different signalling molecules that specifically activate their respective receptors (Tortosa et al., 2001). Thus, we analysed the diversity and distribution of PlcR-PapR quorum sensing system in *B. mycoides* isolates, and determined their pherotypes. This has enabled us to bring the first insight into the distribution of the *B. mycoides* pherotypes among and within the ERIC-types.

We tested the following hypotheses:

- Because of the selective pressure that is induced by the plant rhizosphere, the diversity of bulk soil population of *B. mycoides* will be greater than the diversity of rhizosphere population. Additionally the diversity of rhizosphere population will be greater than that of the endosphere population.

- Isolates from the endosphere will cluster into a clade, as only few *B. mycoides* strains can enter the endosphere of the plant.

- The *plcR* gene, which in *B. cereus* encodes the regulator of its quorum sensing system and shows a high interspecies diversity in other species of *B. cereus* s. l. clade will be also present in *B. mycoides* isolates and will show a comparable diversity. We hypothesized that *plcR* genes of *B. mycoides* will cluster into several putative pherotype groups. The diversity of pherotypes will be greater among bulk soil isolates compared to rhizosphere isolates. We also propose that the *plcR* genes from the endosphere of one plant will be highly homogenous and will code for a single pherotype.

2 LITERATURE REVIEW

2.1 *Bacillus cereus* GROUP

The *Bacillus cereus* group or *B. cereus* sensu lato (s. l.) is a group of bacteria that, at the time of this review (year 2014), comprised seven genetically closely related species: *B. cereus* sensu stricto (s. s.), *B. thuringiensis*, *B. anthracis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides* and *B. cytotoxicus*. The taxonomy of these strains however is complex and univocal and still debatable (Ceuppens et al., 2013).

Representatives of *B. cereus* s. l. are Gram positive, endospore-forming bacteria. They show impressive ecological diversity ranging from their saprophytic lifestyle in soil, to symbiotic (commensal and mutualistic) lifestyles near plant roots. They can also be pathogens in guts of various insects and mammalian hosts, including humans (Drobniewski, 1993; Agaisse et al., 1999; Schnepf et al., 1998; Stenfors et al., 2002; Contzen et al., 2014; Guinebretière et al., 2013; Di Franco et al., 2002).

B. cereus is the causative agent of some food-poisoning syndromes (Drobniewski, 1993). Its ability to cause poisoning has been ascribed to production of several enterotoxins. It is also an important opportunistic pathogen involved in local and systemic infections, which are a consequence of traumatic injuries. Its opportunistic nature is a consequence of its production of extracellular virulence factors, which also include phospholipases and enterotoxins (Drobniewski, 1993).

B. anthracis causes anthrax, a highly infectious, potentially fatal disease, which affects animals as well as humans. This disease begins with the introduction of spores into the body. These can enter through minor abrasions, inhalation or insect bites. After the germination of spores the bacteria multiply and can spread to the regional lymph nodes and then into the bloodstream, where they reach high concentrations, which results in the systemic form of anthrax. The virulence factors that account for the pathogenesis of *B. anthracis* are: an anti-phagocytic capsule, the oedema and lethal toxins (Agaisse et al., 1999)

B. thuringiensis is a well known insect pathogen. Its pathogenic properties are a consequence of the production of δ-endotoxin crystals (also known as Cry proteins) during sporulation. Insect larvae ingest these inclusions, which are than dissolved in the midgut, followed by binding of the toxin to specific receptors on the membrane of midgut and insertion into midgut epithelial cells. This results in formation of trans-membrane leakage pores, which causes cell lysis, and eventual death of the insect. *B. thuringiensis* has been widely used as environmentally friendly control of insect pests (biocontrol) (Schnepf et al., 1998).

B. weihenstephanensis is a psychrotolerant species from the *B. cereus* s. l. It is able to grow at 7 °C. The species is problematic because many *B. weihenstephanensis* strains have the genetic makeup for the production of essential pathogenicity factors, some of which are expressed under laboratory conditions (Stenfors et al., 2002).

B. cytotoxicus is a recently discovered thermotolerant species of *B. cereus* s. l. Its natural habitat is not yet known, but it has usually been isolated from vegetable products and has been also commonly found in agricultural soils. It holds an unusual operon for the *nhe* genes, which encode the components of Nhe, a non-haemolytic enterotoxin. The main factor of its pathogenicity is the cytotoxic activity of the pore-forming cytotoxin K-1. Not all strains are capable of cytotoxic activity, however its poisoning potential is still estimated to be high (Contzen et al., 2014; Guinebretière et al., 2013).

2.2 *Bacillus mycoides*

Bacillus mycoides is the focus of this thesis. Flügge first described the bacterium in 1886 (Flügge, 1886 cit. by Di Franco et al., 2002). As a member of Firmicutes it has a low G+C content and forms spores. When grown in liquid media it usually forms aggregates. Unique characteristic of this species is its asymmetric, hairy and circular colony morphology resembling fungal mycelium, when cultured on agar (fig. 1). Mycelium is made of bundles of filaments curling in clockwise or counter-clockwise direction. The name “mycoides” thus derives from this unique morphological characteristic. The colony morphology of this bacterium has been studied in great detail. Analyses of genes involved in cell wall and cell septum formation (*ftsQ, ftsA, ftsZ* and *murC*) indicated that clockwise and counter-clockwise strains differ genetically but can also switch the direction of the growth. Environmental factors can also influence colony morphology traits, such as branching, growth density (broader or more compact growth) and appearance of cotton-like growth. At temperatures above 35 °C there is a reduction in filament length and above 37 °C, no specific turn direction can be observed (Di Franco et al., 2002; Zhou et al., 2008). It has also been shown that changes in morphology occur when physical force, such as side pressure applied on agar, is used and that it apparently senses holes in agar, which it avoids, by growing around them (Stratford et al., 2013).

B. mycoides has been suggested as a potential biocontrol agent. Its biocontrol potential has been tested in *in vitro* experiments, where *B. mycoides* has been shown to successfully repress the growth of *Pythium mamillatum*, a parasitic fungus that is harmful to cucumber seedlings and causes severe damp-off disease. *B. mycoides* also promoted the growth of the seedlings (Paul et al., 1995). Another biocontrol isolate of *B. mycoides*, Bac J, reduced Cercospora leaf spot (*Cercospora beticola)* in sugar beet both *in vitro* and in experiments done in the field, by inducing systemic acquired resistance in plants (Bargabus et al., 2002).

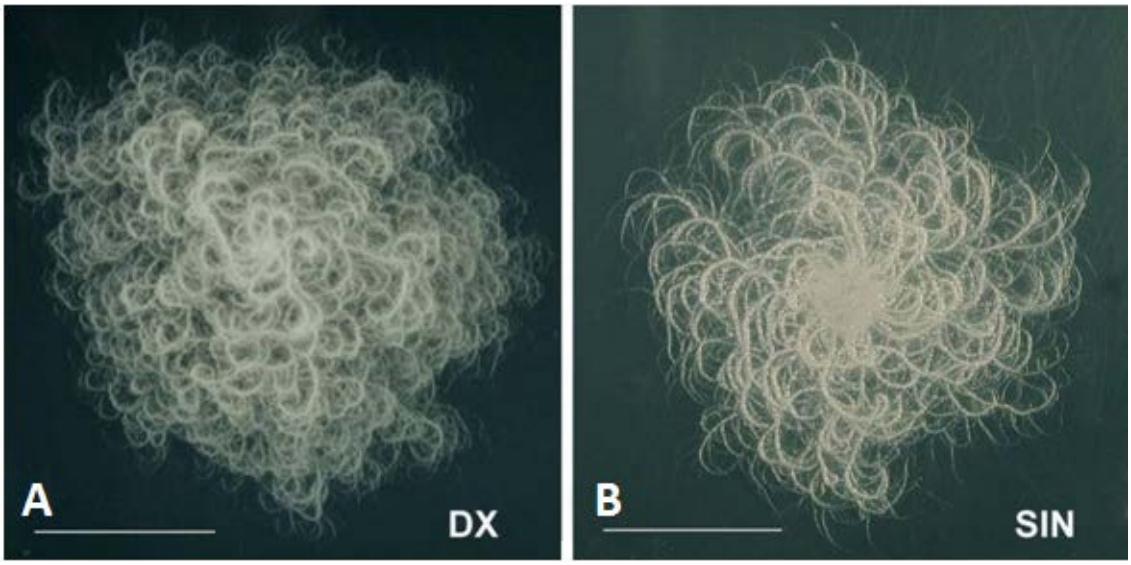

Figure 1: Colonies of environmental isolates of *B. mycoides* (Di Franco et al., 2002). Note the typical fungal-like morphology. **A:** a colony curving clockwise (DX – diextral) **B:** a colony curving counter clockwise (SIN – sinistral). Picture was taken from the top rather than the bottom of agar plate.
Slika 1: Kolonije okolijskih izolatov *B. mycoides* (Di Franco in sod., 2002). Na sliki je lepo opazna tipična, glivam podobna morfologija. **A:** kolonija, ki raste v smeri urinega kazalca (DX– diekstralno) **B:** kolonija, ki raste v nasprotni smeri urinega kazalca (SIN – sinistralno). Slikana je bila zgornja stran kolonij.

2.3 THE GENETICS OF *Bacillus cereus* GROUP

As all of the strains belonging to *B. cereus* s. l. have an important impact on human life and are in general ecologically important it is necessary that they are properly identified and categorised. The species of the group are very closely related with respect to phylogeny, and yet genetically very diverse (Carlson et al., 1994; Helgason et al., 2004; Ticknor et al., 2001). Despite clear phenotypical differences such as mycelial growth of *B. mycoides,* or pathogenicity of *B. anthracis*, many attempts at differentiating and properly defining the *B. cereus* s. l. species on the genetic basis have been made. However, conventional phylogenetic approaches failed to clearly, and undoubtedly define the species.

DNA-DNA hybridization studies done on the whole genomes of *B. cereus, B. thuringiensis and B. antracis* indicated a high level of similarity (Kaneko et al., 1978). Furthermore, the 16S rRNA sequences of *B. cereus, B. thuringiensis, B. anthracis* and *B. mycoides* are almost identical, showing 99% similarity among the species, with changes from four to nine nucleotides (Ash et al., 1991). Analysis of 23 S rRNA of *B. cereus* s. s. and *B. anthracis* again failed to increase the resolution power, which suggested that ribosomal genes are not sufficient for taxonomic analysis of this group (Ash and Collins, 1992).

Genomic mapping, using a combination of multi locus enzyme electrophoresis (MLEE), restriction enzyme digestion profiling (REDP) and pulsed field gel electrophoresis (PFGE) was used to try and distinguish *B. thuringiensis* strains from *B. cereus* strains but the approach proved to be unsuccessful. The comparison of *B. cereus* type strain (*B. cereus* ATCC 14579) with a specific *B. thuringiensis* strain showed that, apart from missing a specific enzyme restriction site and having two additional ones, the *B. thuringiesis* strain was largely identical to the *B. cereus* type strain. The *B. thuringiensis* strain however, contained a plasmid, which carries the insecticidal toxin-coding gene (Helgason et al., 2000; Helgason et al., 2004). PFGE combined with MLEE was applied in an attempt to distinguish *B. cereus* and *B. thuringiensis* strains. The study showed that in spite of the high variability between the groups and within the groups, no consistent specific differences could be found that would define the two species. Thus, the study presented another argument to consider these species as one (Carlson et al., 1994). Multi enzyme electrophoresis in combination with sequencing of nine chromosomal genes has also been applied in an attempt to differentiate *B. cereus* s. s. and *B. thuringiensis*. The results again encouraged the suggestion that the bacteria belong to the same species (Helgason et al., 2000). Another multi locus sequence typing (MLST) analysis was performed on seven housekeeping genes of *B. cereus*, *B. thuringiensis*, *B. weihenstephanensis* and *B. anthracis*, which again showed high genetic diversity of the group, yet failed to clearly define the individual species (Helgason et al., 2004).

As we can see, the taxonomic status of this bacterial group is very problematic. The difficulties are best portrayed with problems of defining *B. thuringiensis* and its differentiation from *B. cereus* s. s. The problem with this species is that the only thing clearly defining *B. thuringiensis* is the ability to produce insecticidal toxin for which the gene is coded on a plasmid. Thus a strain of *B. thuringiensis* that has lost the plasmid, or is lacking the gene for the toxin is indistinguishable from *B. cereus* s. s. and in turn, a *B. cereus* s. s. cell that would receive the plasmid would become indistinguishable from *B. thuringiensis* (Carlson et al., 1994; Carlson et al., 1996; Helgason et al., 2000).

2.4 ERIC-PCR

Enterobacterial repetitive intergenic consensus PCR, or ERIC-PCR, is a variation of genomic fingerprinting techniques collectively known as rep-PCR, that were first used to distinguish between enteric bacteria (Versalovic et al., 1991). Prokaryotic organisms have interspersed repetitive sequences in their genomes that are targeted by this technique. The interspersed elements are conserved in several genera and the rep-PCR method is based on the observation that outwardly facing oligonucleotide primers, which are complementary to these interspersed repeated sequences, amplify differently sized DNA fragments representing sequences between these elements. The fragments are then separated by gel electrophoresis, which results in DNA fingerprint patterns specific for individual bacterial strains and can be used to distinguish them (Versalovic et al., 1991; Versalovic et al.,

1994). We chose rep-PCR technique because it has successfully been used before in *B. ereus* s. l. (Cherif et al., 2003; Kim et al., 2002). The method can also be used to distinguish other Gram positive and Gram negative bacteria and even viruses and eukaryotic organisms (Gillings and Holley, 1997).

2.5 QUORUM SENSING AND THE PlcR-PapR SYSTEM OF *Bacillus cereus* GROUP AND ITS DIVERSITY

Bacteria use cell-to-cell communication that is dependent on cell density, called quorum sensing. Quorum sensing systems of Gram positive and Gram negative bacteria are different, especially in the context of their signals (Miller and Bassler, 2001). Gram negative bacteria communicate via small molecules called autoinducers, which are compounds consisting of a homoserine lactone ring and a fatty acid residue, usually in the form of acyl-homoserine-lactone; however, some species can apply different forms of autoinducers (Parsek and Greenberg, 2000; Pesci et al., 1999; Williams, 2007). In Gram positive bacteria these signal molecules are short oligopeptides (Bouillaut et al., 2008; Håvarstein et al., 1995). Quorum sensing systems are responsible for many community-driven processes. The first such process observed was bioluminescence in *Vibrio fischeri.* Other processes, which can be regulated by quorum sensing systems, can be of survival essence, such as biofilm formation (Davies et al., 1998; Hammer and Bassler, 2003) and sporulation (Solomon et al., 1996). These processes also include events that involve exchange of genomic material such as genetic competence for transformation (Håvarstein et al., 1995) or plasmid transfer (Fuqua and Winans, 1994). Quorum sensing signal is also important in orchestrating virulence (Grenha et al., 2013).

There are usually several “languages” or groups of signalling molecules in the scope of a quorum sensing system in several Gram positive bacteria (Tortosa et al., 2001) that are known as pherotypes. These endow bacteria with a pherotype-specific communication system, where a cell of one pherotype can usually communicate only with cells of the same pherotype. However, there is a possibility of weak cross-talk in some cases (Bouillaut et al., 2008; Slamti and Lereclus, 2005; Štefanič and Mandić Mulec, 2009).

Representatives of the *B. cereus* s. l. group encode the PlcR-PapR quorum sensing system (Lereclus et al., 1996; Slamti and Lereclus, 2002), where PlcR is the receptor protein, and PapR is the signalling peptide. Based on structure analysis studies, it was suggested that PlcR belongs to the RNPP family, along with Rap, NprP and PrgX regulators. It shows week similarity with PreL and NprA, the regulators of the protease gene in *Lactobacillus* and *Bacillus stearothermophilus*. PlcR is a 34 kDa pleiotropic transcriptional regulator of different genes. It positively regulates its own expression and, in *B. cereus,* the transcription of at least 15 genes potentially involved in bacterial virulence (e.g. phospholipase C, haemolysins, and protease). Deletion of the *plcR* gene in *B. cereus* and

B. thuringiensis significantly reduced haemolytic activity and virulence of the bacterium in insect larvae (Slamti and Lereclus, 2002). The *plcR* regulon in *B. thuringensis* controls the expression of 45 genes encoding secreted proteins like bacteriocins, enterotoxins, hemolysins and degradative enzymes that may all add to the pathogenesis of this bacterium (Bouillaut et al., 2008; Gohar et al., 2008; Lereclus et al., 1996; Slamti and Lereclus, 2002).

The physiologically active form of PlcR is a homoduplex of two PlcR protein molecules (fig. 2). Binding of the PapR signal peptide causes slight conformational changes, unveiling of the DNA binding site and consequentially promotes binding of the regulator to the PlcR-box (fig. 2) (Bouillaut et al., 2008; Declerck et al., 2007; Grenha et al., 2013). PlcR-box is a highly conserved palindromic region with the sequence TATGNANNNNTNCATA positioned not far from the PlcR regulated genes (Agaisse et al., 1999).

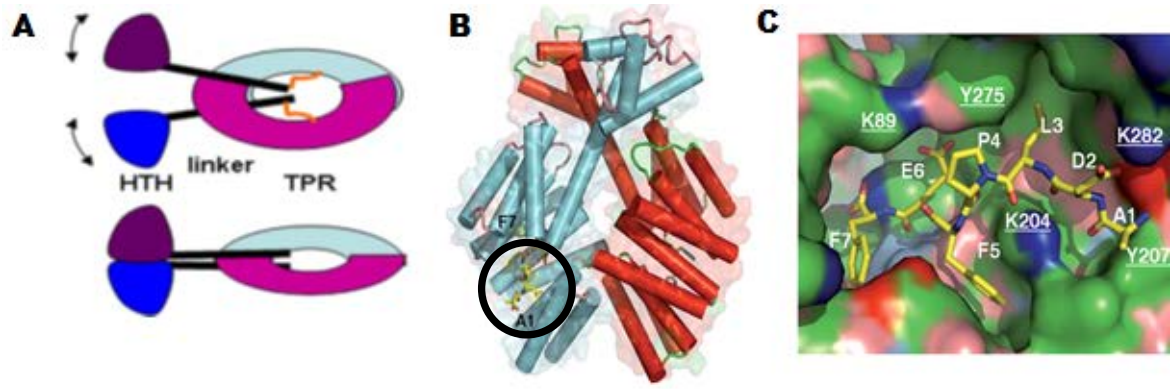

Figure 2: Molecular models of PapR7I peptide bound to PlcRI receptor. **A:** A schematic depiction of the conformational change of PlcR homoduplex after PapR signal has activated it (Declerck et al., 2007). **B:** Two protomers of the PlcR (receptor) dimer are coloured in red and light blue. Helices are represented by tubes, within the transparent molecular surface. PapR7I (heptapeptide signal) is shown as a stick model (circled-A1) (Bouillaut et al., 2008). **C:** PlcR is shown as molecular surface and PapR7I as a stick model (Bouillaut et al., 2008).
Slika 2: Molekularni modeli peptida PapR7I vezanega na receptor PlcRI. **A:** Shematski prikaz konformacijske spremembe homodupleksa PlcR po aktivaciji s PapR (Declerck et al., 2007). **B:** Dimer dveh protomerov PlcR (receptor), en je obarvan rdeče in drugi svetlo modro. Heliksi so prikazani z valji razporejenimi znotraj molekularne površine, ponazorjene s prosojnim obarvanjem. PapR7I (heptapeptidni signal) je prikazan kot palični model molekule (obkroženo) (Bouillaut et al., 2008). **C:** PlcRI je prikazan kot molekularna površina in PapR7I kot palični molekularni model (Bouillaut et al., 2008).

The PlcR-PapR system is encoded by the *plcR-papR* locus. The *papR* gene is located 70 bp downstream of the *plcR* gene (fig. 3). The gene encodes a 48 amino acid peptide (Agaisse et al., 1999). We now know that PapR polypeptide is secreted from the cell and then re-imported into the cell by oligonucleotide permease (Opp) (Gominet et al., 2001). What is left in the cell is a processed active form of the PapR heptapeptide, which was initially

believed to be a pentapeptide (Slamti and Lereclus, 2002), but has later been discovered that the actual active physiological form is a heptapeptide (Bouillaut et al., 2008; Slamti and Lereclus, 2005).

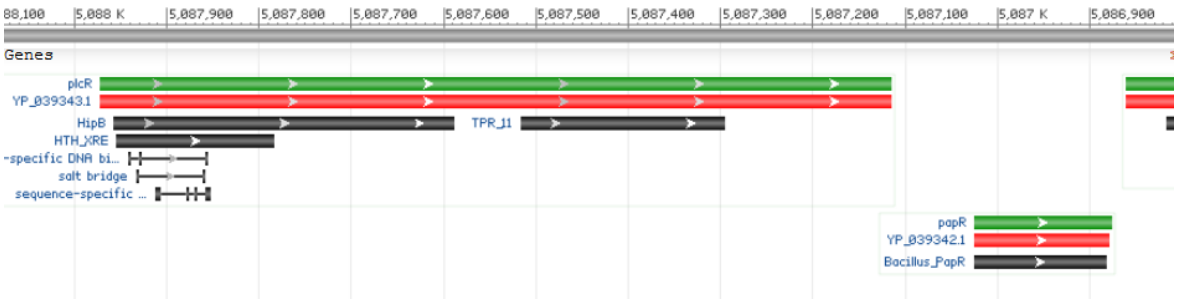

Figure 3: The structure of *plcR-papR* locus of *Bacillus thuringiensis* serovar konkukian strain 97-27. The *papR* is positioned 70bp downstream of the *plcR* gene (NCBI, 2014).
Slika 3: Struktura lokusa *plcR-papR* bakterije *Bacillus thuringiensis* serovar konkukian sev 97-27. Gen *papR* se nahaja 70bp nižje od gena *plcR* (NCBI, 2014).

Polymorphism, observed in the PlcR-PapR quorum sensing system, has been associated with different language groups (pherotypes) within the *B. cereus* species. The phylogenetic tree (fig. 4) that was based on the 29 PlcR amino acid sequences of *B. cereus* s. l. showed that, of the four groups, the fourth was the most distant from the other groups, having 27.7% divergence. Slamti and Lereclus (2005) also discovered that each pherotype forms its own specificity group based on specificity in recognition between signals and receptors. However, evidence of cross-pherotype communication was also uncovered (Slamti and Lereclus, 2005). Later studies with heptapeptide signals indicated that quorum sensing activation is specific; however, cross-pherotype communication was still possible and could not be ruled out (Bouillaut et al., 2008).

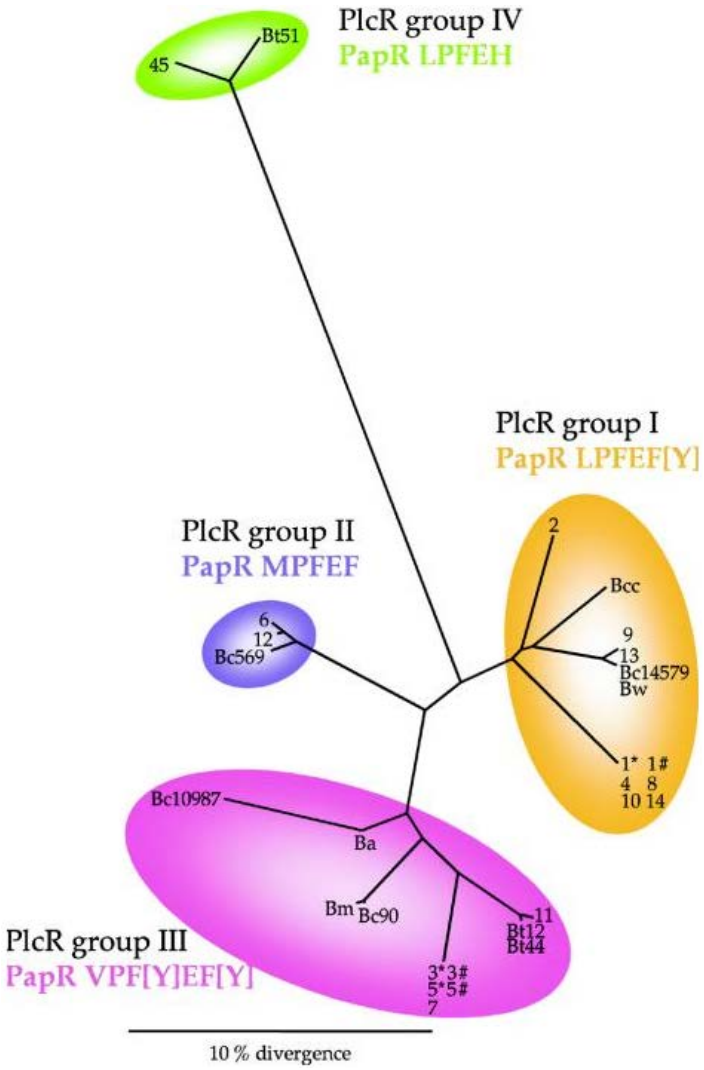

Figure 4: A similarity dendrogram that depicts four groups of PlcR pherotypes with their respective signal pentapeptides indicated in the corresponding colour (Slamti and Lereclus, 2005).
Slika 4: Dendrogram podobnosti, ki prikazuje štiri skupine PlcR ferotipov s pripadajočimi, enako obarvanimi signalnimi pentapeptidi (Slamti and Lereclus, 2005).

3 MATERIALS AND METHODS

3.1 MATERIALS AND SOLUTIONS

All materials used are written in tab. 1. The materials are divided by their application i.e. process in which they were mainly used.

Table 1: A list of all reagents and media components used.
Preglednica 1: Seznam vseh reagentov in sestavin gojišč, ki smo jih uporabili.

Material	**Product details**
	Media
Agar:	Micro Agar, product no. M1002, Duchefa Biochemie
Peptone:	Bacto™ Peptone, product no. 211677, BD Biosciences
Meat extract:	Meat extract, product no. 103979, Merck Millipore
	Bacterial isolation and glycerol stocks
Na_2HPO_4:	di-Sodium hydrogen phosphate, product no. 106586, Merck Millipore
KH_2PO_4:	Potassium dihydrogen phosphate, product no. 104873, Merck Millipore
Glycerol:	Glycerol, BioUltra, for molecular biology, anhydrous, ≥99.5% (GC), product no. 49767, Sigma Aldrich
Glass beads:	Glass beads, unknown manufacturer diameter
	DNA extraction
Tris:	Tris, product no. 10708976001, Roche
EDTA	Ethylenediaminetetraacetic acid disodium salt dihydrate, product no. E5134, Sigma-Aldrich
NaCl	Sodium Chloride, product no. 106404 Merck Millipore
Lyzozyme	Lyzozyme, product no. 105281, Merck Millipore
RNase	RNase, product no. 1119915, Roche
Phenol	Phenol Solution, product no. P4557, Sigma-Aldrich
Chloroform:	Chloroform, product no. 5100245, Merck Millipore
Ethanol, absolute	Alcohol, Anhydrous, Reagent, product no. 9401, J. T. Baker
Phase lock gel:	Phase Lock Gel, product no. 2302800, 5 Prime
PCR-grade water:	Thermo Scientific™ HyClone™ Water, Molecular Biology Grade, product no. SH30538, Fisher Scientific
	PCR amplification
Taq polimerase kit:	GoTaq DNA Polymerase, product no. M3005, Promega
	Gel Electrophoresis
TAE buffer:	50X TAE Buffer (Tris-acetate-EDTA), product no. B49 Thermo Scientific
High definiton Agarose:	Agarose, High resolution, DNase, RNase, NICKase, none detected, product no. A4718, Sigma-Aldrich
DNA electrophoresis standard:	GeneRuler DNA Ladder Mix, product no. SM033, Fermentas, Thermo Scientific
Staining dye:	GelRed, Biotium

3.2 SOLUTIONS AND ENZYMES

Saline solution

For preparation of 500 ml of saline solution (0.9% (w/v) NaCl), 4.5 g of NaCl was dissolved in 300 ml dH_2O and topped with dH_2O to the final volume of 500 ml. The solution was autoclaved and stored at room temperature.

TES (Tris - Ethylenediaminetetraacetic acid (EDTA) - Sodium chloride) buffer

For preparation of 500 ml of TES, 1 M Tris-HCl and 0.25 M EDTA had to be prepared first.

1 M Tris-HCl: 3.63 g of Tris base was dissolved in 25 ml of dH_2O. The pH was adjusted to 8.0 with HCl and the solution topped with dH_2O to the final volume of 30 ml.

0.25 M EDTA: 1.46 g of EDTA base was dissolved in 20 ml of dH_2O.

TES: 25 ml of 1 M Tris-HCl solution was mixed with 250 ml of dH_2O, then 10 ml of 0.25 M EDTA solution was added, and finally 1.5 g of NaCl was dissolved in the solution. After the components were well mixed, the solution was topped with dH_2O to the volume of 500 ml.

1x SDS (Phosphate Buffered Saline) buffer

For 1 l of PBS, 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 were dissolved in 800 ml of dH_2O and then topped to final volume of 1 l with dH_2O. The pH was adjusted with HCl to 7.4. The solution was autoclaved.

10% SDS (Sodium dodecyl sulphate) solution

For 50 ml 10% (w/v) SDS, 5 g of SDS powder was dissolved in 40 ml of dH_2O, mixed with magnetic stirrer, dH_2O was then added to the final volume of 50 ml. The solution was autoclaved.

Lyzozyme

To prepare 50 mg/ml of the enzyme solution, we dissolved 75 mg of the enzyme powder in 1.5 ml of PCR grade water contained in 2 ml microcentrifuge tube. The suspension was vortexed until the powder was visibly dissolved. The solution was stored in -20 °C.

Media

Nutrient broth medium (NB) (700 ml) was prepared by adding 2.1 g of meat extract and 3.5 g of peptone to 500 ml dH_2O. After components dissolved, the medium was diluted to final volume of 700 ml with dH_2O. To prepare nutrient agar (NA), we added 10.5 g of agar

to pre-prepared NB and mixed until particles were hydrated. The medium was than autoclaved.

3.3 METHODS AND PROCESESS

3.3.1 Plant and soil samples

3.3.1.1 Sampling site

The samples were obtained in Netherlands, the province Drente in the Midden-Drenthe municipality, town Eurosinge, the exact location being 52°49'49.34"N, 6°37'15.97"E (fig. 5, fig.6). They were obtained from a potato field where many different cultivars of the potato plant are cultivated.

Figure 5: The satellite image of the experimental field (yellow pin) located in the northern part of Netherlands (Google Earth, 2014).
Slika 5: Satelitski posnetek lokacije vzorčenja (rumena bucika) na severu Nizozemske (Google Earth, 2014).

Figure 6: A close up of the experimental field where sampling was performed (Google Earth, 2014)
Slika 6: Bližji pogled na lokacijo eksperimentalnega polja, kjer smo vzorčili (Google Earth, 2014)

3.3.1.2 Bulk soil samples

We sampled bulk soil at the end of June 2013. The samples were taken from two different garden beds that were 15 meters apart from each other. In one garden bed, the cultivar planted was Miranda and in the other, the cultivar planted was Seresta. The garden beds were named after the cultivar (Miranda garden bed, Seresta garden bed). The sampling sites of bulk soil were one meter apart from each other. We took 13 samples from each garden bed, 26 samples of bulk soil altogether. The sampling scheme is presented in fig. 7. The soil was stored in sterile 50 ml Falcon tubes and transported at environmental temperature. When brought to the laboratory, samples were immediately stored at 4 °C and used later in that day.

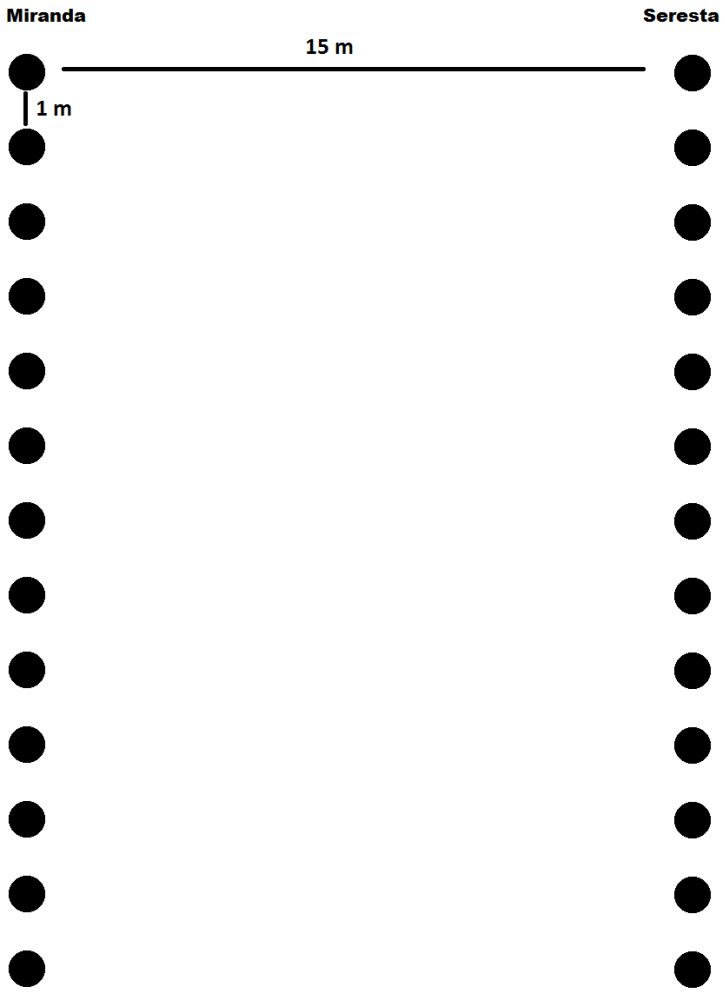

Figure 7: Sampling scheme for the bulk soil.
Slika 7: Shema vzorčenja tal.

3.3.1.3 Plant samples

Plant sampling was performed in the second week of July in the year 2013. We sampled two different potato cultivars, Miranda and Seresta. For each cultivar we sampled three plants (roots with adhered soil). Most of the soil adhered to roots was shaken off at the sampling site and the thin layer of soil that remained attached to the roots was preserved. The samples were stored in clean, non-bleached paper bags and transported to the laboratory at environmental temperature. Samples were then processed immediately. A portion of the sample that was not used was stored at 4 °C in the same paper bags in which the samples were brought in. These remaining samples were not used and were later discarded.

3.3.2 Isolation of *B. mycoides* strains

3.3.2.1 Preparation of bulk soil samples

B. mycoides are endospore-forming bacteria and the isolation of such bacteria routinely involves heat treatment, which eliminates vegetative cells, but preserves endospores.

1 g of wet soil per sample was suspended in 1 ml of saline solution in a microcentrifuge tube and mixed vigorously by vortexing. The sample was then placed in a heating block at 80 °C for 15 minutes. After the heat treatment, the samples were vortexed again and re-suspended in 4 ml of sterile saline solution in Falcon tubes. Samples were then vigorously vortexed and 100 µl of prepared suspension was spread onto two agar plates per sample. Plates were incubated at 28 °C for 48 hours.

3.3.2.2 Preparation of rhizosphere samples

Rhizosphere is a thin layer of soil adhered to plant roots, that remains attached even after thorough shaking of the roots. We weighed approximately 0.20 g of roots with adhered rhizospheral soil. The roots were put in a 15 ml Falcon tube with glass beads and PBS buffer. The tubes were placed horizontally in a shaker for 20 minutes. After treatment, the roots appeared to be completely clean, without a trace of rhizospheral soil. The roots were stored into sterile Petri dishes at 4 °C. The samples were processed the same day. The rhizosphere solution was heat treated the same way as the bulk soil samples. After the heat treatment, the samples were vigorously vortexed and 100 µl of prepared suspension was spread onto two agar plates per sample. Plates were incubated at 28 °C for 48 hours.

3.3.2.3 Preparation of root samples for endophyte isolation

For endophyte isolation, we surface sterilized the roots by washing them with 70% ethanol and then placing them into a sterile Petri dish with 70% ethanol for 10 minutes. To remove ethanol, we transferred the roots were to sterile saline solution for 10 minutes. After washing, we fine cutted the roots with a sterile scalpel into pieces of approx. 3-5 mm in length. For each cut, the blade was sterilised by dipping the blade into rubbing alcohol and flaming it and thus reducing the chance of cross-contamination.

After cutting, we placed the root pieces on nutrient agar with sterile tweezers. The tweezers were also sterilised before transferring each piece onto an agar plate. We prepared two plates per sample.

3.3.2.4 Obtaining isolates

The colonies with the proper morphology (the unique, fungal-like colonies) were picked with sterile toothpicks and re-inoculated onto a fresh agar plate, which was then incubated at 28 °C for 48 hours.

After 48 hours the individual isolate colonies were scraped off with an inoculation loop and streaked to purity onto an agar plate. The streak plate method was performed three times for each isolate, which were then considered to be a pure culture. After that, the isolates were ready for stock preparation and DNA extraction.

3.3.2.5 Glycerol cell stock culture preparation and DNA extraction

Cells were grown in 10 ml NB medium in 50 ml Falcon tubes on a shaking incubator at 28 °C, 250 rpm for 24 hours. Media was inoculated with single hyphae, scraped from the colony. Some colonies were very strongly adhered to the surface of the agar and therefore they were transferred to NB with some agar residue. Liquid cultures were then used for stock preparation or for DNA extraction.

3.3.2.6 Glycerol stock cultures

It is good laboratory practice to preserve all newly obtained isolates. The protocol for the glycerol stock preparation was obtained from the official web site of The Ohio State University, Department of Horticulture and Crop Science (Stockinger, 2013). We adjusted the protocol to prepare 25% glycerol stock cultures.

After 24 hours of incubation the cultures were aliquoted into 1.5 ml screw cap microcentrifuge tubes with autoclaved glycerol which we had prepared earlier. The final concentration of the glycerol was 25%. The suspension was well vortexed and labeled. The strain stocks were stored at -70 °C. We prepared three stock cultures for each isolate.

After the stocks were prepared we tagged each stock with the following tag: DTJS 2013/initial of the cultivar/number of the plant/initial of the sphere/number of the isolate. Example: DTJS 2013/M1E-1 (tab. 2).

Table 2: Explanation of the tag abbreviations of the isolates.
Preglednica 2: Obrazložitev okrajšav pri oznakah izolatov.

Abbreviation	**Explanation**
M	Miranda potato cultivar
S	Seresta potato cultivar
1, 2, 3	The plant number (three plant replicates)
E	Endosphere
R	Rhizosphere
B	Bulk soil

The isolates were stored at the University of Groningen, Faculty for Mathematics and Life sciences, Microbial Ecology Department and at the University of Ljubljana, Biotechnical Faculty, Department for Food Sciences, Chair of Microbiology.

3.3.3 DNA extraction

First we centrifuged 2 ml of the overnight culture for 2 minutes at 16000 g, after removal of supernatant, the cells were re-suspended in 300 µl TES buffer and then transferred to a 1.5 ml microcentrifuge tube. After that we added 6 µl of lysozyme and 2 µL of RNase and incubated the mixture in a thermo block or in a water bath at 37 °C for 30 minutes. After the incubation we added 33 µl of 10% SDS and again incubated the suspension in thermo block at 50 °C for 2 hours. Next, phenol/chloroform DNA extraction was performed.

We used Phase Lock Gel® at the phenol and chloroform step. The gel physically stabilizes ("locks") the interphase and thus prevents mixing of the organic and water phase, minimizing the contamination with the proteins from the interphase and maximizing the yield.

After lysis, 200 µl of phenol was added to the lysis mix (308 µl + pellet) and the tube was mixed gently but thoroughly by hand. This step removes the proteins from the DNA and denatures them. Samples were centrifuged for 2 min at top speed to separate the two phases after which the top phase was carefully removed and transferred to a sterile microcentrifuge tube. Next, 200 µl of chloroform was added to remove the residual phenol and remaining proteins that we might have had transferred from the phenol step. Again, the sample was centrifuged for 2 minutes at the top speed. After centrifugation the top phase was carefully transferred to a sterile microcentrifuge tube and DNA was precipitated with 2 volumes of 96% ethanol that was cooled to -20 °C. The tube was gently shaken and then centrifuged for 10 min at the top speed. After centrifugation, the ethanol was very carefully discarded as not to lose the DNA pellet. DNA was washed with 500 ml of 70% ethanol that was cooled to -20 °C. Samples were incubated on ice for 15 min and then centrifuged for 10 min at the top speed. We carefully discarded the ethanol, performed another quick spin and removed the remaining ethanol with a pipette. The DNA pellet was dried in a vacuum concentrator to evaporate the residual ethanol, after that the dry pellet was re-suspended in 100 µl PCR grade water.

DNA was extracted from isolates obtained from Miranda garden bed including microscale isolates MB-3 and MB-13, and the bulk soil isolates from Seresta garden bed. We did not extract DNA from Seresta isolates.

3.4 GENOMIC FINGERPRINTING

3.4.1 ERIC-PCR protocol

The PCR mix of 25 µl contained 1× concentrated reaction buffer without $MgCl_2$, we added 2.5 mM of $MgCl_2$, 0.1 mM of dNTPs, 0.5 µl of each primer (tab. 3), 4 U of *Taq* polymerase, and PCR grade water. 1 µl genomic DNA was added to each reaction.

The PCR program consisted of initial denaturation step at 95 °C for 5 minutes, 30 cycles of denaturation step at 95 °C for 1 minute, annealing step at 45 °C for 1 minute and elongation step at 72 °C for 1 minute. The reaction ended with final elongation step at 72 °C for 10 minutes.

We ran two PCR reactions for each DNA sample in two individually prepared PCR mixtures to ensure the reproducibility of the patterns. As temperatures used for annealing were slightly lower than normally applied (Versalovic, 1994) we performed six independent PCR reactions on three randomly chosen isolates to verify the reproducibility of the method (results, fig. 9).

Table 3: Primers used in ERIC-PCR experiments.
Preglednica 3: Oligonukleotidni začetniki, ki smo jih uporabili pri ERIC-PCR reakcijah.

Primer name	Primer sequence	Reference
ERIC1R:	5'-ATGTAAGCTCCTGGGGATTCAC-3	(Versalovic et al., 1994)
ERIC2:	5'-AAGTAAGTGACTGGGGTGAGCG-3'	(Versalovic et al., 1994)

3.4.2 Agarose gel electrophoresis

We prepared 2% agarose gel following the protocol of the manufacturer found on their official web site (Sigma Aldrich, 2014). The size of the tray for gel preparation and running was 15 cm in length and the PCR duplicates were ran alongside each other.

The electrophoresis ran at 70 V, 250 mA for 5 hours and the agarose gels were stained with GelRed solution for 20 min and washed in dH_2O. After that the gel was photographed under UV light and the image was saved in a digital form for further analysis.

3.4.3 Analysis of genomic fingerprints

For the analysis of photographs we used the BioNumerics software (Applied Maths, 2014). The software offers several bioinformatics tools in one program and is thus very useful. Among these, there is also a tool that enables the analysis of patterns obtained by methods such as SDS-PAGE, PCR-DGGE, PFGE and rep-PCR. The power of the program is in its ability to compare large numbers of gel pictures by normalizing the background, and the

position of bands in relationship to the position of bands of the DNA ladder standard. The program is then able to calculate the similarity of the patterns obtained, by applying different mathematical algorithms. It then uses calculated data to draw similarity dendrograms.

To obtain data from a gel picture, the user must first have a proper digital format of the picture, which is usually already standard in imaging equipment, so the images are already compatible with the program. In the very first stage of the program, the user must define the value for background reduction, which is than constant throughout the analysis of the images. The images must be cropped and the curvature, or "smiling", of bands must be defined. In the next step, the electrophoresis lanes must be defined, followed by tagging of DNA ladder bands (so called external standard). This step is required only on the first image, on all the following images, the ladder must be re-tagged so that the program notes the discrepancy and adjusts the image of the gel accordingly. Internal standard can also be defined and for this purpose we used the bands which were repeatedly present in a gel and were undoubtedly on the same position. In the next step, the user must tag the bands. We used automatic band recognition with default settings. With these settings we were able to avoid most of misrecognized bands, but in turn also lost auto recognition of some of the weaker bands. Some manual adjustments had to be made for misinterpreted bands, which occurred due to dust particles, bubbles or other artifacts in gels, additionally when a single band was tagged two times, we had to remove the second tag. Apart from misinterpreted artifacts, there were only few such adjustments.

The program converts data into band presence/absence matrix, based on which a similarity matrix can be calculated. The similarity matrix is than the basis for similarity dendrogram construction. For calculating the similarity matrix, we applied a Jaccard algorithm, and for construction of the similarity tree, we used the UPMGA algorithm. The settings were: 0.5% optimization, 1.5% tolerance in band matching.

We defined ERIC-type (OTU) as an isolate or a group of isolates, which form a cluster at 81% similarity mark on the dendrogram. This is an arbitrary limit, based on the shape of the dendrogram and is a limit, which provides us with the most information about our sample.

3.4.4 Isolate diversity/richness estimation

To estimate the diversity of the isolated bacteria we used Shannon-Wiener index (H'). According to Ge et al. (2008), it is one of the most widely used equations for diversity estimations. We used the following formula:

$$H' = -$$

In this formula E stands for number of operational taxonomic units (OTU-s) in the sample. We named OTUs as ERIC-types, P*i* is the proportion of ERIC-types *i* and n_i is the number of representatives of ERIC type *i*.

3.4.5 Quorum sensing gene amplification and sequencing

To explore the quorum sensing diversity of our strains, we amplified a segment (*plcR* gene) of *plcR-papR* locus which codes the PlcR-PapR quorum sensing system in *B. cereus* s. l. (Lereclus et al., 1996; Slamti and Lereclus, 2002). We randomly chose isolates of which *plcR* genes we later amplified and sequenced. In addition, two sets of microscale soil isolates were also sequenced. The reason for randomly choosing a sub-population of isolates was to reduce the scope of analysis.

For the *plcR-papR* locus amplification we used the primers in tab. 4, which we designed manually. The primers were designed with the help of Anna Oślizło.

Table 4: Primers used with *plcR* gene amplification.
Preglednica 4: Oligonukleotidni začetniki, ki smo jih uporabili pri pomnoževanju gena *plcR.*

Primer name	**Primer sequence**
Forward primer (plcRF_Bm)	5’-CGAATTCACATTATTGTAGTGG-3’
Reverse primer (plcRR_Bm)	5'-GGAGTAACATCCAAACGTC-3’

The end volume of PCR mixture was 25 µl. The reagents in the mixture contained reaction buffer without $MgCl_2$, which was 1× concentrated, 2 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.5 µM of each *plcR* primer, 0.025 of *Taq* polymerase and PCR grade water. An amount of 0.5 µl DNA was added to each reaction.

The PCR program consisted of initial denaturation step at 95 °C for 2 min, 30 cycles of annealing at 55 °C for 30 sec and elongation at 72 °C for 1 min. The reaction ended with final elongation step of 72 °C for 10 min.

The PCR products were sent for sequencing to a commercial facility . The sequencing service of our choice is performed with ABI3730XL as well as ABI3700 sequencers, and reads of up to 1100 bp in length are guaranteed by the company (Macrogen Inc., The Netherlands). Our main focus was *plcR* gene, which in *B. cereus* s. l. species is 858 bp or 864 bp long (depending on the pherotype group).

3.4.6 Sequence analysis

The acquired sequences were analyzed with the software MEGA5 (Tamura et al., 2011). We aligned several publically available *plcR* sequences (tab. 5) that were also used by Slamti et al (2005) along with our sequences (tab. 9). After aligning, the excess regions

outside the gene were discarded, resulting in sequences of 858 bp (pherotype PlcR-3) or 864 bp (pherotype PlcR-4) in length. When aligning, computing similarity matrices and constructing similarity dendrograms, we used amino acid translations of sequences. Settings used for aligning were changed for "Pairwise Alignment": gap opening penalty: 3.0; gap extension penalty: 1.8. Based on this alignment we constructed a similarity dendrogram which enabled us to determine the pherotypes of our isolates. Another dendrogram was constructed only with sequences obtained in this work. For drawing the dendrograms and calculating similarity matrices we used neighbor-joining method and Jones-Taylor-Thornton (JTT) model respectively.

Table 5: A list of reference *B. cereus* s.l. strains used to determine pherotypes of our strains (tab. 9).
Preglednica 5: Seznam referenčnih sevov skupine *B. cereus* s. l., ki smo jih uporabili pri določanju ferotipov naših izolatov (pregl. 9).

Strain name	**Acession no.**
PlcR group I	
Bacillus thuringiensis, serotype H 1	AJ582632
Bacillus thuringiensis, serotype H 4	AJ582635
Bacillus thuringiensis, serotype H 8	AJ582669
Bacillus thuringiensis, serotype H 9	AJ582670
Bacillus thuringiensis, serotype H 13	AJ582674
PlcR group II	
Bacillus thuringiensis, serotype H 6	AJ582637
Bacillus thuringiensis, serotype H 12	AJ582673
Bacillus cereus strain 569	AY195601
PlcR group III	
Bacillus mycoides	AY776141
Bacillus cereus isolate Bc90	AY776145
Bacillus thuringiensis, serotype H 7	AJ582638
Bacillus thuringiensis, serotype H 11	AJ582672
PlcR group IV	
Bacillus thuringiensis isolate Bt51	AY776146
Bacillus thuringiensis serovar roskildiensis	AJ583466

Sequences referenced in this table were used in a study by Slamti et al. (2005).
Seve navedene v tej preglednici so v svoji študiji uporabili Slamti in sod.(2005).

4 RESULTS

4.1 ISOLATION OF *B. mycoides*

We obtained 350 *B. mycoides* isolates from rhizosphere and endosphere of three Miranda and three Seresta cultivar potatoes and from the bulk soil samples of their respective garden beds. On agar plates, inoculated with bulk soil samples, microscale soil samples and rhizosphere samples, we observed many different types of colonies. However, the colonies of *B. mycoides* were very easily recognisable, as they had the recognisable fungal-like growth with broad diameter relative to other colonies. We isolated one isolate per bulk soil sample (13 isolates per garden bed, together 26 isolates). In addition to these isolates, we also obtained **microscale** isolates, from three Miranda and three Seresta bulk soil samples (samples 3, 8 and 13 of both garden beds). We obtained 14 isolates per bulk soil sample of which all of the 14 isolates were isolated from the same gram of soil, for example: isolate MB-3 was isolated from the same gram of soil as isolates MB-3/2 to MB-3/15. The number of isolated strains per sample is presented in table below (tab. 6) and the typical morphology of isolated strains can be found on fig. 8.

Table 6: Number of isolated strains per sample (habitat).
Preglednica 6: Število izoliranih sevov na vzorec (okolje).

cultivar	Miranda			Seresta		
plant number	1	2	3	1	2	3
endosphere	20	20	20	20	20	20
rhizosphere	20	20	20	20	20	20
garden bed	Miranda			Seresta		
bulk soil	13			13		
microscale sample 3	14			14		
microscale sample 8	14			14		
microscale sample 13	14			14		

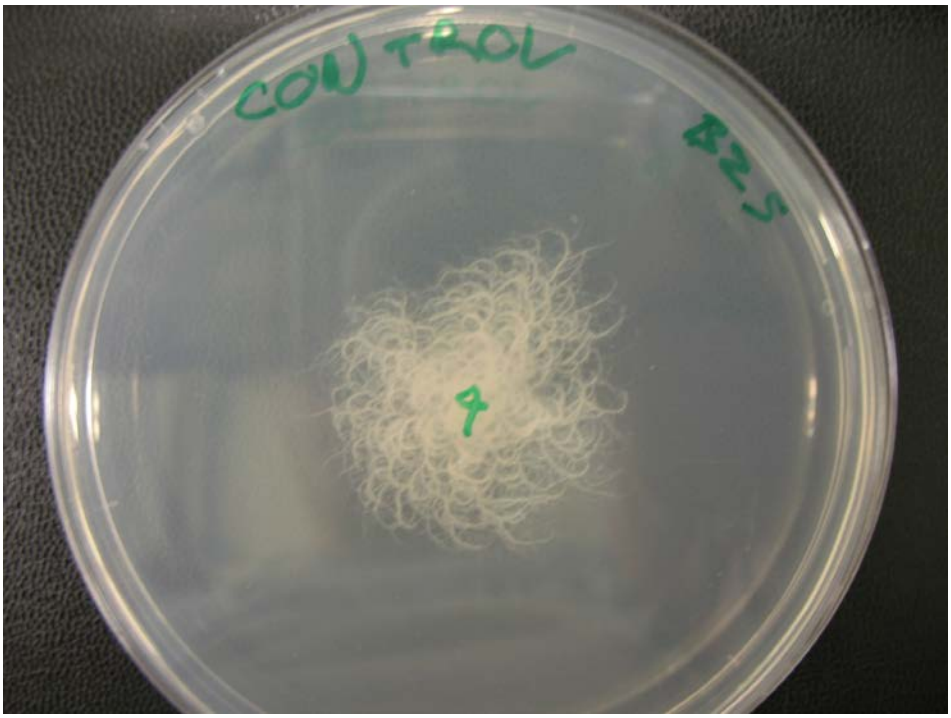

Figure 8: The unique, fungal-like colony morphology of *B. mycoides*.
Slika 8: Edinstvena, glivam podobna morfologija kolonij bakterije *B. mycoides*.

4.2 ERIC-PCR

To distinguish between different *B. mycoides* isolates, we performed ERIC-PCR analysis followed by hierarchical clustering.

This way, we analyzed 60 isolates from the Miranda endosphere, 60 from Miranda rhizosphere and 12 from bulk soil of the garden bed. The remaining 13 bulk soil isolates originated from Seresta garden bed. Therefore, we analyzed 146 *B. mycoides* isolates. The isolate MB-3 did not produce a fingerprint and was excluded from further analysis.

Initial experiments involved standard annealing temperatures used in ERIC-PCR (Versalovic et al., 1991) and did not produce satisfactory results. Thus, we lowered the annealing temperature from 52 °C proposed by Versalovic et al. (1991) to 45 °C. We then tested the reproducibility of the modified method. To this aim we analyzed three isolates with six individually-prepared PCR reactions. As can be observed in fig. 9, the test showed distinct and highly reproducible ERIC patterns for each strain, suggesting that the robustness of the reaction was not affected by lower annealing temperature and that the reaction can still discriminate between strains of *B. mycoides*.

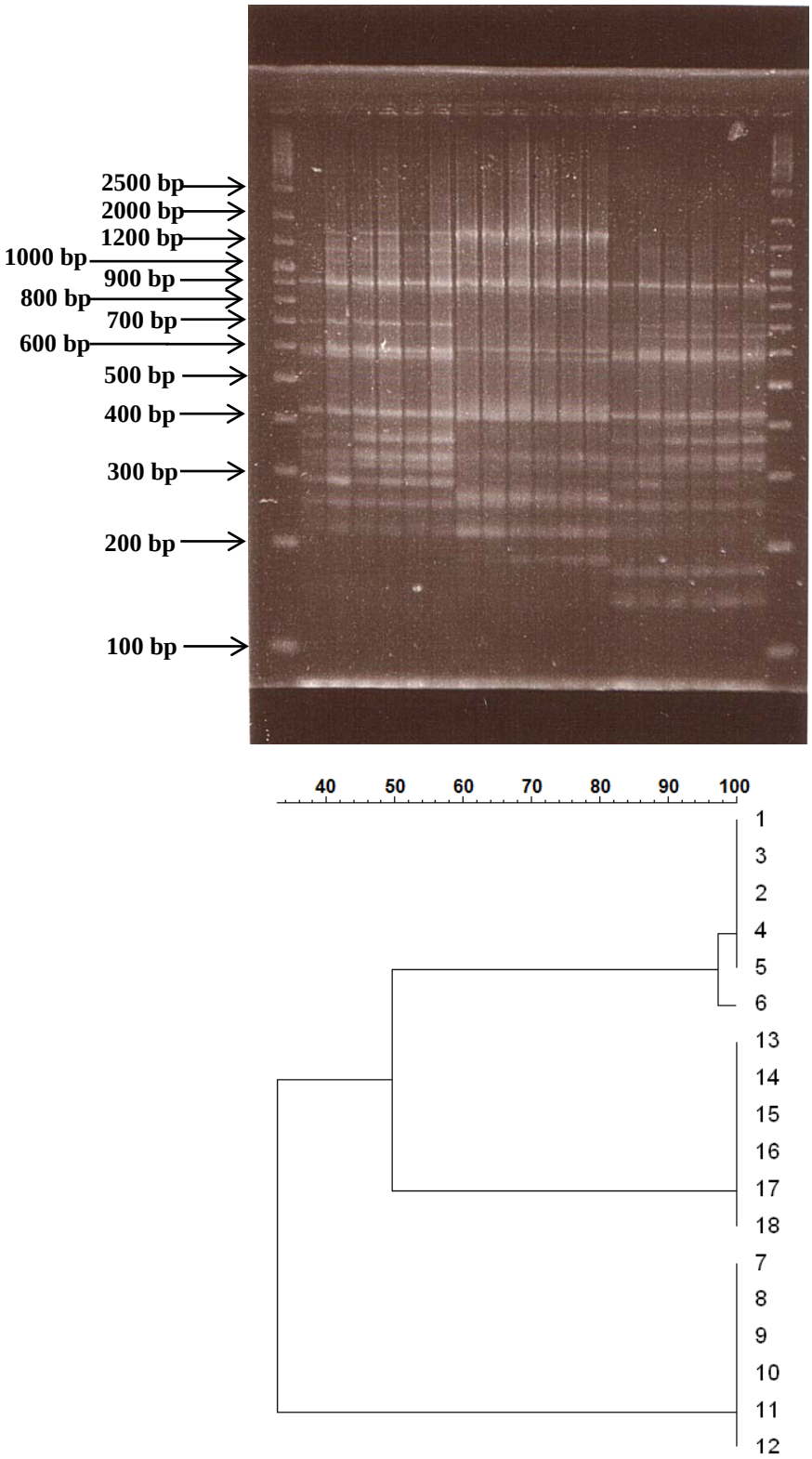

Figure 9: ERIC-PCR reproducibility test. **A**: Electrophoretogram of the reproducibility. The samples on the gel are: 1-6: M1R-12; 7-12: M2E-6; 13-18: MB-1. B: Dendrogram showing similarity of the fingerprints.
Slika 9: Test ponovljivosti reakcij ERIC-PCR. A: Elektroforetogram testa ponovljivosti. Vzorci na gelu: 1-6: M1R12; 7-12 M2E-6; 13-18: MB-1. B: Dendrogram, ki prikazuje podobnost prstnih odtisov.

The ERIC-PCR fingerprints of our isolates were complex. Fingerprints contained 6 to 15 bands, with usual number being in range of 10 to 13 bands. We observed clear differences among samples, which were highly reproducible. The bands ranged in size from around 5000 bp to just above 100 bp. However, only bands with sizes above 150 bp and below 4000 bp were used for further analyses. The reason for this approach is the low visability of these bands and consequentially a high possibility of missing the bands. The ERIC-PCR reaction of soil isolate MB-3 did not produce a visible fingerprint (data not shown) and was thus excluded from further analysis.

4.2.1 Hierarchical clustering

The ERIC-PCR fingerprinting data were analysed by a computer-assisted hierarchical cluster analysis using BioNumerics software. Using this approach, we constructed a similarity dendrogram (fig. 10) and then defined operational taxonomic units (OTU) which we termed ERIC-types, which include isolates with patterns that express similarity value higher than the arbitrarily defined cut-off value of 81% similarity. Therefore, patterns that showed 81% or higher similarity grouped into the same OTU. Based on these criteria, we distinguished 24 OTUs that we will refer to as ERIC-type groups. The obtained data were also used to calculate the diversity indices for the populations. The reason we chose 81% cut-off value is because the ERIC-types as defined by this cut-off value best correlates with clusters that can be observed on the dendogram (fig. 10). A stricter cut-off value (e.g. 82% value) would cause higher calculated diversity as ERIC-type group Q would be split into two groups. On the other hand, a looser value (e.g. 80%) would cause lower diversity as two large groups (C and D) would merge into one.

The ERIC-PCR dendrogram (fig. 10) indicated the existence of four large ERIC-type groups (C, D, E, R), comprised of 16 or more isolates, with E being the largest. ERIC-type groups, F, Q consisted of six or more isolates, nine groups comprised at least two isolates (A, B, G, H, K, O, P, S, U) and the remaining nine ERIC-type groups were each represented with one isolate (I, J, L, M, N, T, W, X).

Within most ERIC-type groups, there were sub-groups of isolates that showed 100% similarity in their fingerprints. The exceptions were clusters A, B, K and P. Few isolates expressed a very low level of similarity to other fingerprints, with the most distant being the SB-4 fingerprint which showed only 25% similarity, followed by MB-4 and MB-5 which were both 41.67% similar to their most distant relatives. The majority of the strains's fingerprints on the dendrogram exhibited at least 72% similarity to other fingerprints.

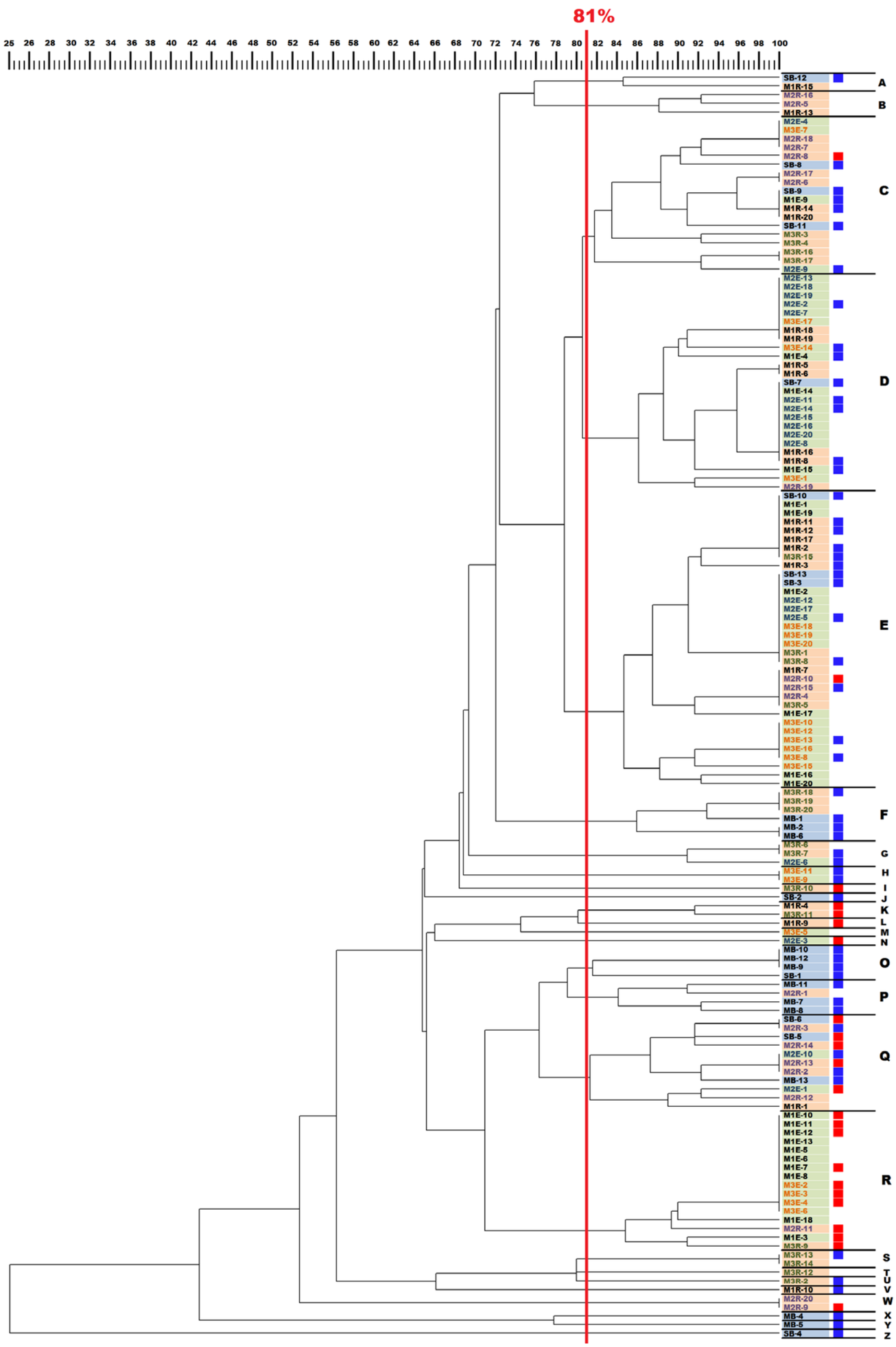

Figure 10: ERIC-PCR dendrogram, based on genomic fingerprinting results. The dendrogram was constructed using BioNumerics software. The red vertical line marks the 81% similarity cut-off. The lines on far right separate ERIC-types (OTUs), and the letters represent the assigned ERIC-type, which was later used in tab. 7. The blue and red squares next to strain names represent different pherotypes: blue squares: pherotype PlcR-3; red squares: pherotype PlcR-4.

Slika 10: Dendrogram vzorcev ERIC-PCR, ki temelji na genomskih prstnih odtisih. Dendrogram je bil narisan s programsko opremo BioNumerics. Rdeča črta označuje mejo 81 % podobnosti. Črne črte na desni med seboj ločujejo ERIC-tipe (operativne taksonomske enote), črke pa predstavljajo oznake ERIC-tipa, ki smo jih uporabili v preglednici 7. Rdeči in modri kvadratki poleg imen izolatov predstavljajo različne ferotipe: modri kvadratki: ferotip PlcR-3; rdeči kvadratki: ferotip PlcR-4.

Table 7 : Cross-tabulation of ERIC-PCR clustering results.
Preglednica 7: Navzkrižni prikaz rezultatov ERIC-PCR.

Habitat	Name/ plant no.	ERIC-type group																							
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
Bulk soil	Sereste	1		3	1	3					1					1		2							1
	Miranda						3									3	3	1					1	1	
Rhizosphere	1	1	1	2	6	6						1	1					1			1				
	2		2	5	1	3											1	5	1			2			
	3			4		4	3	2		1		1							1	4					
Endosphere	1			1	3	6													**10**						
	2			2	**11**	3		1							1			2							
	3			1	3	**9**			2					1					4						

The cells contain numbers of representatives of an ERIC-type in individual samples.
V celicah je zapisano število predstavnikov posameznega ERIC-tipa.

Only nine ERIC-types were detected among the 60 **endosphere** isolates, suggesting a more uniform population in comparison to the **rhizosphere soil** where 16 ERIC-types were detected among the same number of strains. The 25 isolates from **bulk soil** clustered into 12 ERIC-types, suggesting a highly diverse population in this habitat.

In the ERIC-type groups D, E and R, endosphere isolates were predominant. Interestingly, within each group the isolates from an endosphere of a different plant were most abundant. In plant no. 1 most isolates were in ERIC-type R and represented 50% of all isolates, in plant no. 2 the dominant type was D, which represented 55% of all isolates, and in plant no. 3 the dominant type was E which represented 45% of all isolates.

In the **rhizosphere soil** the distribution of different ERIC-types was more even, with the highest frequency (six per type) of isolates found in ERIC-types D and E. Additionally, isolates from plant no. 2 fell in two main ERIC-type groups: C and Q, each containing five rhizosphere representatives. Isolates from plant no. 3 clustered mainly into groups C, E and S with four rhizosphere representatives per ERIC-type. Interestingly, none of the ERIC-types prevalent in rhizosphere soil appeared to be dominant in the endosphere. It is important to note here that the endosphere isolates we obtained via surface ethanol treatment of the roots are not necessarily endophytes. The reason for such possibility is the resistance of bacterial spores to various concentrations of ethanol for extended periods of time (Thomas, 2012). Therefore, it is quite possible that the isolates we obtained are actually the cells, which developed from the spores produced by the dominant ERIC-type that was present on the root surface.

4.2.2 Shannon-Wiener diversity index

We calculated Shannon-Wiener diversity index as a mean to estimate the diversity of *B. mycoides* populations. Due to the disproportionate nature of our samples that differed in number of isolates included in the sample (number of bulk soil isolates, included in analysis, was much lower (25 isolates) than in rhizosphere and endosphere set (60 isolates each)) we performed computer assisted random re-sampling, using Microsoft Excel. Based on randomly selected isolates we then performed hierarchical clustering analysis and constructed dendrograms, which we then used to calculate the diversity indices. We repeated the procedure three times for both the endosphere and rhizosphere population, to ensure the consistency and validity of the results. The resulting dendrograms can be found in the annex.

To calculate diversity indices we defined OTUs at the 81% similarity threshold. The results are presented in tab. 8, and the calculations are presented in the annex. The bulk soil diversity index is the same in all three comparisons, as it was calculated based on the same set of isolates, while we see differences in Shannon-Wiener diversity indices for the other two sets of isolates. Our results show that the diversity is lowest for the endosphere isolates and highest for bulk soil isolates, with rhizosphere isolates showing an intermediate diversity.

Table 8: Results of Shannon-Wiener diversity index calculations.
Preglednica 8: Rezultati izračunov Shannon-Wienerjevega indeksa.

	Endosphere	Rhizosphere	Bulk soil
Shannon-Wiener diversity index	1.4580	2.0652	2.3243
	1.7313	2.1688	2.3243
	1.2971	1.8124	2.3243

The calculations are based on analysis of dendrograms based on ERIC-PCR fingerprints of 25 randomly selected isolates from each habitat. The cut-off value used to define the OTUs was 81%, the same as in defining OTUs of all isolates. Index of bulk soil isolates is the same on all three occasions because the same isolates were used for the analysis.
Izračuni temeljijo na analizi dendogramov, ki temeljijo na prstnih odtisih ERIC-PCR 25 naključno izbranih izolatov iz vsakega habitata. Meja, s katero smo definirali operativno taksonomsko skupino, je bila 81% podobnost. Indeks izolatov iz tal je v vseh treh primerih enak, saj so pri izračunih bili uporabljeni isti izolati.

4.3 DIVERSITY OF QUORUM SENSING GENE FRAGMENTS

We sequenced 114 *plcR* gene fragments (*plcR* gene group 1, 2 and 3: 858 bp; group 4: 864 bp) from randomly selected isolates originating from endospheres or rhizospheres. Out of 114 sequences, 101 sequences were of sufficient quality for further analysis (tab. 9). The *plcR* sequences were aligned and compared to sequences of different *B. cereus* s. l. species published in Slamti and Lereclus (2005) to determine the pherotype group of our isolates. The previously published sequences comprising the *B. cereus* s. l. group clustered into four PlcR groups. The *plcR* genes from our collection of *B. mycoides* isolates clustered only in two groups: the PlcR group III and PlcR group IV (termed PlcR-3 and PlcR-4 respectively, for the purpose of this thesis) as is depicted in fig. 11.

Table 9: A list of randomly selected strains isolated from three different environments used in PlcR analysis.
Preglednica 9: Seznam naključno izbranih izolatov pridobljenih iz treh okolji, ki smo jih uporabili pri analizi PlcR.

Names of isolates									
Endophytes (E)			Rhizosphytes (R)			Bulk soil isolates (B)			
M1E	M2E	M3E	M1R	M2R	M3R	MB	MB-3/	MB-13/	SB
3	1	1	2	2	2	1	8	2	1
4	2	2	3	3	7	2	9	3	2
7	3	3	4	8	8	3	10	4	3
9	5	4	8	9	9	4	11	5	4
10	6	8	9	10	10	5	12	6	5
11	9	9	10	11	11	6	13	7	6
12	10	11	11	13	13	7	14	8	7
15	11	13	12	14	15	8	15	9	8
	14	14	14	15	18	9		10	9
								11	10
									11
									12
									13

The isolates originate from from endosphere (E) and rhizosphere (R) of Miranda potato cultivar plants and from bulk soil (B) from the field planted with Miranda and Seresta cultivar. The table contains isolate names, of which *plcR* genes were successfully sequenced.
Izolati izvirajo iz endosfere (E) in rizosfere (R) kultivarja Miranda in iz tal zasajenega s tem kultivarjem in kultivarjem Seresta (B). Preglednica vsebuje imena izolatov, katerih *plcR* geni so bili uspešno sekvencirani.

4.3.1 Computer-assisted cluster analysis

Analysis of the 101 *plcR* sequences with MEGA software resulted in the dendrogram shown as fig. 11. As we can observe, all of the sequenced isolates cluster into two out of four known pherotypes (Slamti and Lereclus, 2005). The majority (89%) of analysed isolates clustered into PlcR-3 and the rest into PlcR-4 pherotype group. The largest part (72.60%) of PlcR-3 as well as of PlcR-4 (89%) representatives exhibited 100% identity between them at the protein level. All *plcR* genes from the bulk soil isolates (including microscale soil samples and bulk soil samples) that clustered into PlcR-3 were identical, with the exception of SB-4. The rest of the pherotype PlcR-3 representatives originated from rhizosphere soil and the endosphere. These showed rather low divergence, ranging from 0.004% to 0.075%. The *plcR* sequences of pherotype PlcR-4 were mostly 100% identical, with the exception of the sequences of isolates M3R-11, M1R-4 and M2R-10. The dissimilarity within the pherotype groups is evident in fig. 12.

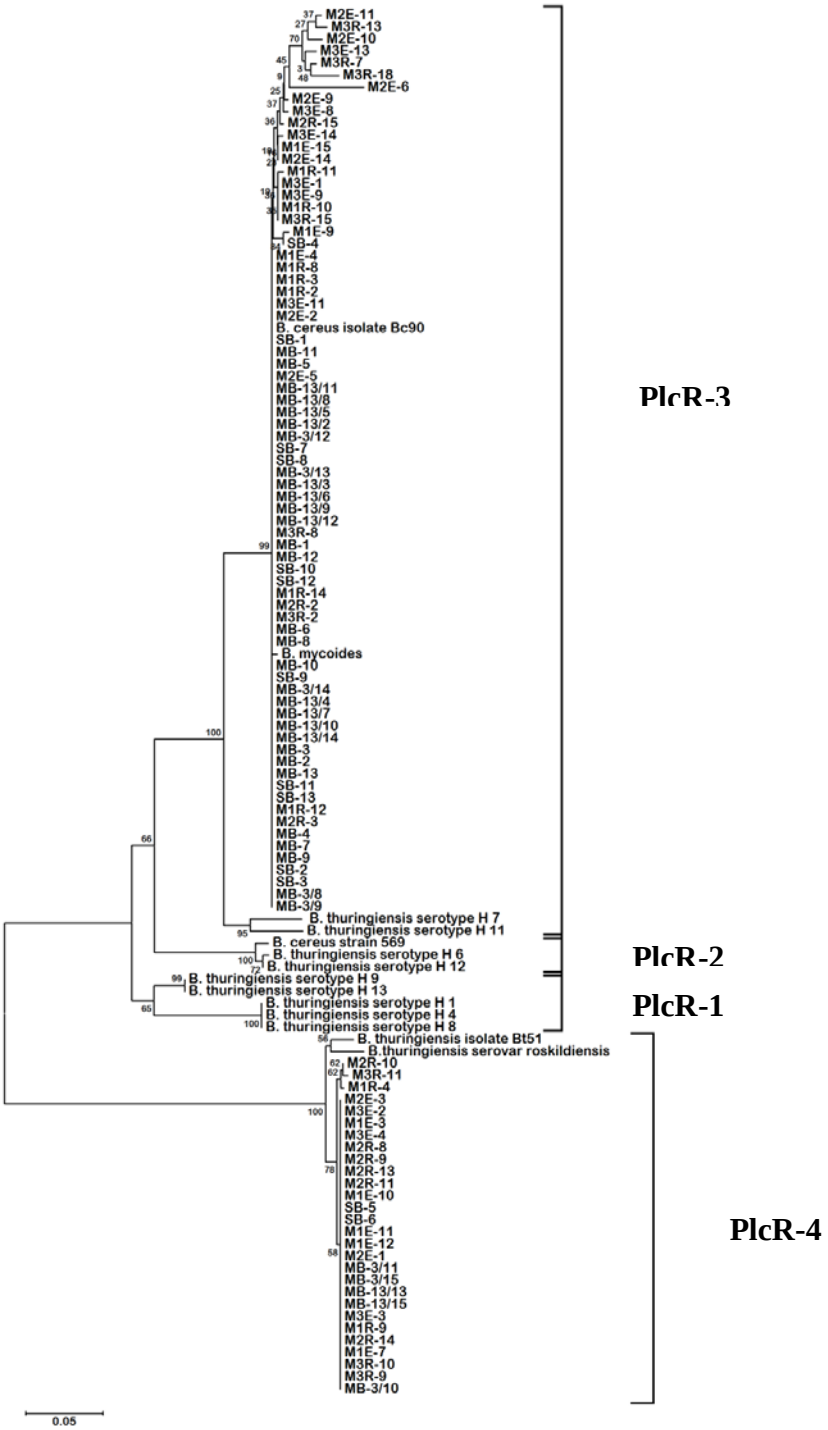

Figure 10: Similarity dendrogram depicting clustering of *plcR* genes with reference genes. The latter were used in the study done by Slamti and Lereclus (2005). The similarity dendrogram shows clustering of quorum sensing system genes of our isolates into known pherotypes. M: Miranda cultivar, S: Seresta cultivar, B: bulk soil, R: rhizosphere, E: endosphere, 1, 2, 3: plant number.

Slika 11: Dendrogram podobnosti, ki prikazuje grupiranje *plcR* genov z referenčnimi geni. Slednji so bili uporabljeni v razskavi, ki sta jo izvedli Slamti in Lereclus (2005). Dendrogram prikazuje grupiranje genov sistemov zaznavanja kvoruma naših izolatov v štiri znane ferotipe. M: kultivar Miranda, S: kultivar Seresta, B: zemlja, R: rizosfera, E: endosfera, 1, 2, 3; številka rastline.

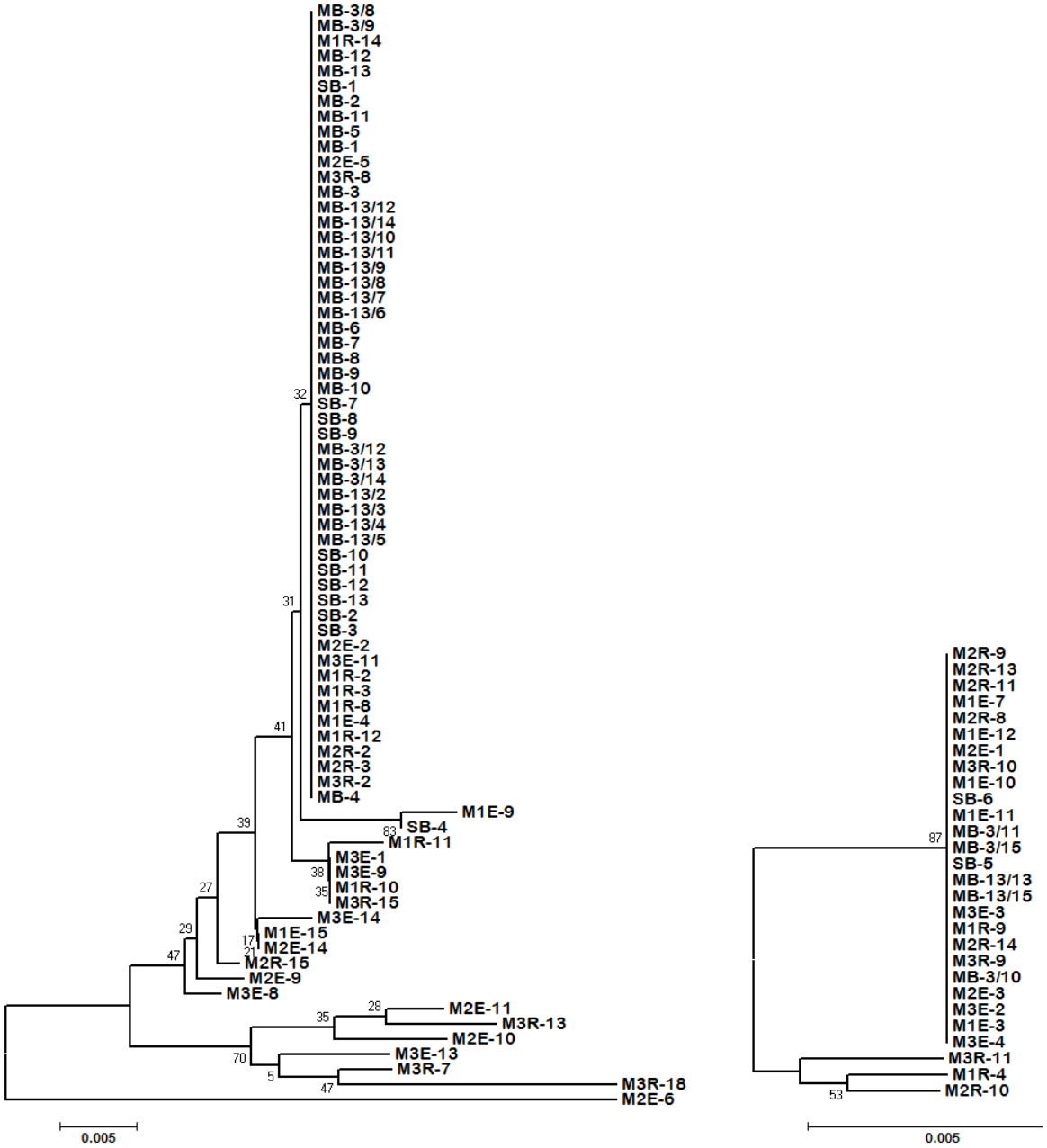

Figure 11: Similarity dendrograms of *plcR* genes. **A:** Similarity dendrogram of *plcR* genes belonging to PlcR-3 pherotype. **B:** Similarity dendrogram of *plcR* genes belonging to PlcR-4 pherotype. M: Miranda cultivar, S: Seresta cultivar, B: bulk soil, R: rhizosphere, E: endosphere, 1, 2, 3: plant number.
Slika 12: Dendrograma podobnosti za gene *plcR*. **A:** Dendrogram podobnosti genov *plcR* , ki sodijo v PlcR-3 ferotip. **B:** Dendogram podobnosti genov *plcR*, ki sodijo v feroptip PlcR-4. M: kultivar Miranda, S: kultivar Seresta, B: zemlja, R: rizosfera, E: endosfera, 1, 2, 3; številka rastline.

The distribution of the PlcR-3 pherotype between rhizosphere and endosphere isolates was identical, with 16 representatives from each habitat. Similarly, the pherotype PlcR-4 had 10 rhizosphere and 11 endosphere representatives (fig. 13).

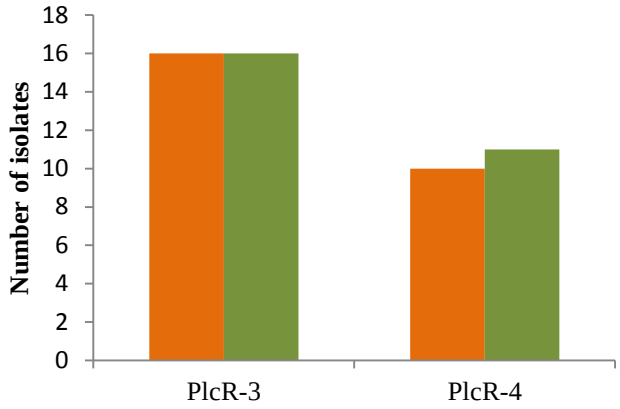

Figure 12: Bar chart of cumulative distribution of pherotypes between rhizosphere and endosphere of three Miranda cultivar potato plants. Orange bar represents rhizosphere, green bar represents endosphere
Slika: 13: Stolpični diagram kumulativne distribucije ferotipov med rizosfero in endosfero treh rastlin krompirja kultivarja Miranda. Oranžni stolpec predstavlja rizosfero, zeleni stolpec predstavlja endosfero

The distribution of pherotypes between endospheres and rhizospheres for individual plants (fig. 14) shows a slightly different picture, where in each habitat one pherotype was dominant (mostly PlcR-3, except in groups E1 and R2) with plant no. 1 and plant no. 2 showing opposite distribution of pherotypes.

It is important to emphasise here, that bacteria which were isolated from, and co-exist within rather small distances clustered into both pherotype groups. Such isolates include the ones isolated from rhizosphere of one plant and especially the ones isolated from endosphere of one plant. They also include strains, isolated from the same microscale sample.

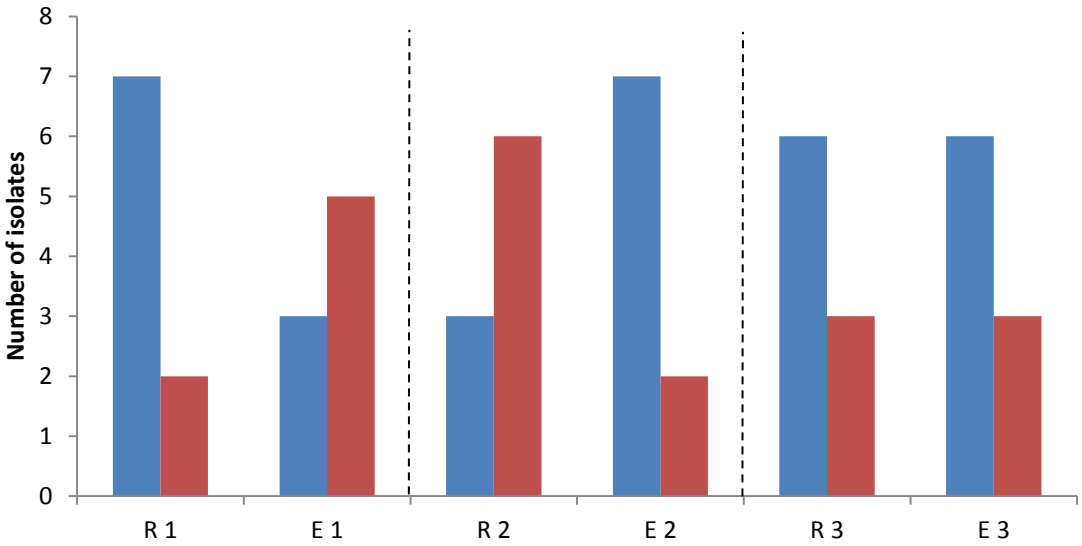

Figure 13: Bar chart of proportional distribution of pherotypes amongst rhizospheres and endospheres of each potato plant. Blue: pherotype PlcR-3; red: pherotype PlcR-4. R: rhizosphere; E: endosphere; 1, 2, 3: plant number.
Slika 14: Stolpični diagram proporcionalne distribucije ferotipov med rizosfero in endosfero vsake posamezne rastline krompirja. Modra: ferotip PlcR-3; rdeča: ferotip PlcR-4. R: rizosfera, E: endosfera, 1, 2, 3; številka rastline.

4.3.2 Correlation of pherotypes and ERIC-types

On the ERIC-PCR dendrogram (fig. 10), pherotypes of isolates are marked with coloured squares next to their name (red and blue squares). The predominant pherotype among isolates, the PlcR-3 (89% of isolates) was, expectedly, predominant among the ERIC-type groups. However, in the ERIC type R all sequenced *plcR* genes belonged to the pherotype PlcR-4. In contrast, the ERIC type Q consisted of isolates of both pherotypes in almost equal proportions (four PlcR-3 genes and five of PlcR-4 genes).

It is also worth noting that two isolates M2R-10 (PlcR-4) and M2R-15 (PlcR-3) in ERIC group E, and five isolates from ERIC type Q (SB-6 (PlcR-4), M2R-3 (PlcR-3), M2E-10 (PlcrR-3), M2R-13 (PlcR-4) and M2R-2 (PlcR-3)) had identical ERIC-PCR pattern within the ERIC type, but different pherotypes. This suggests that similarity in overall genome is not connected to phylogenetic relatedness in the locus encoding the PlcR-PapR quorum sensing system, which represents the social relatedness of isolates.

5 DISCUSSION

Microbial diversity in soil has an important functional role. Bacteria affect soil structure, nutrient cycling, soil water retention and also have an important role in plant growth, plant disease and pest control (Pimentel et al., 1997). In this study, we focused on the diversity of *Bacillus mycoides* populations isolated from three different habitats: bulk soil from a potato field (garden beds), rhizosphere soil and the endosphere of potato plants. Using ERIC-PCR, we analysed and compared the diversity of *B. mycoides* populations in these three habitats. We also analysed the diversity of the PlcR-PapR quorum sensing system by analysing the *plcR* genes. The PlcR-PapR quorum sensing systems are known to diverge into pherotypes in other species from the *B. cereus* s. l. clade, thus we addressed whether the *plcR* genes are also diverse in *B. mycoides*, how many pherotypes they represent and how these are distributed in relation to the three analysed habitats.

5.1 *Bacillus mycoides* COMMUNITY STRUCTURE AND DIVERSITY

To observe the structure of *B. mycoides* populations and estimate the diversity in endosphere, rhizosphere and bulk soil we applied the repetitive sequence-based PCR method (also known as rep-PCR) where we used primers named ERIC. This approach enabled us to "scan" regions of the entire genome of an isolated organism (Versalovic et al., 1991; Versalovic et al., 1994). This is especially important when comparing isolates of a species such as *B. mycoides*, which belongs to a taxonomic group of highly similar species, namely *B. cereus* senso lato. These species show little variation at the level of the 16S rRNA gene sequence (Ceuppens et al., 2013).

Based on the dendrogram derived from ERIC-PCR fingerprinting data we defined the OTU similarity cut-off value at 81% similarity, a parameter, which we used to calculate Shannon-Wiener diversity indices and define OTUs (ERIC-types). The ERIC-types as defined by cut-off value of 81% best correlate with clusters that can be observed on the dendogram. A stricter cut-off value (e.g. 82% value) would cause higher calculated diversity as ERIC-type group Q would be split into two groups. On the other hand, a looser value (e.g. 80%) would cause lower diversity as two large groups (C and D) would merge into one. This approach revealed that all *B. mycoides* isolates cluster into 24 ERIC-types. Bulk soil isolates (Miranda and Seresta) were present as ERIC-types in all but one large group (group R) and in some of the minor groups as well. Diversity index calculations were congruent with wide ERIC-type distribution, as the estimated diversity was the highest in bulk soil population (H = 2.3243), lower for rhizosphere (average H = 2.0155) and the lowest for endosphere (average H =1.4955) populations of *B. mycoides*. These results could be explained by habitat selective conditions. Plants release vast amounts of simple to highly complex root exudates, which represent energy sources for bacteria and other microorganisms. These exudates consist of carbon molecules, such as primary metabolites and sugars and they contain secondary metabolites, enzymes (Bais et al., 2006;

Bertin et al., 2003) and other highly desirable compounds, to which bacteria are attracted. The exudates also include signal molecules that enable cross-kingdom communication with susceptible organisms (Lugtenberg et al., 2002) and can contain compounds called phytoalexins as well (Reva et al., 2004). The latter compounds create a selective environment, which may attract only a sub-population of the bacterial pool, while blocking others; they are a plant's way of selecting and maintaining a desirable rhizosphere microflora (Bais et al., 2006). Another form of selection in rhizosphere may reside in the opportunity for high metabolic activity and thus for high numbers of organisms (Berg et al., 2014) that are competing for substrates. In our case, the diversity of the **endosphere** population was even lower in the rhizosphere, and the populations appeared to be uniform compared to the rhizosphere populations. In previous studies addressing bacterial endophytes, similar observations were made (Berg et al., 2005, Edwards et al., 2015). We also observed that each plant's endosphere was populated by a different dominant ERIC-type which represented 50%, 55% and 45% of all isolates obtained from plant no.1, no.2 and no.3, respectively. The fact that each plant's endosphere was populated by a different dominant ERIC-type suggests that the dominance could occur as an event of opportunity, as the process of establishing it could be stochastic. As described above in this paragraph, the plant creates a selective environment in its rhizosphere and as such, the presented environment could be suitable for a particular strain of *B. mycoides* more than other strains, and could therefore enable it to dominate that environment. Once dominant, the strain could then invade the plant, as it has little competition with other strains. The dominant ERIC-types in the endosphere however, were not dominant in the rhizosphere soil, which is consistent with previous findings on bacterial populations in plants (Berg et al., 2005; Germida et al., 1998). It is possible that factors specific for the endosphere (determinants of invasion success) enable the specific ERIC-types to establish dominance. In addition to the rhizosphere itself being a selective environment for bacteria, the rhizoplane (root surface) could be the main selector for the entrance into the endosphere, as it is a very specific and highly-demanding habitat for bacteria. The ability to adhere to the rhizoplane might therefore be necessary for entering the root endosphere (Edwards et al., 2015; Germida et al., 1998), which may at least in part explain why ERIC-types that were in minority in rhizosphere could prosper in endosphere. It is important to note that we cannot guarantee that our isolates from the endosphere are actually endophytes, as ethanol is not toxic for endospores of *Bacillus* sp. (Thomas, 2012). Therefore, the obtained isolates could have originated from spores that were produced by the strains on the rhizoplane.

It would be interesting to explore, what the mechanisms for plant invasion are in *B. mycoides* and which specific factors have a role in excluding certain strains of *B. mycoides* at the plant-entry level, while enabling others to dominate and eventually enter the plant's endosphere.

5.2 QUORUM SENSING

We analysed 101 *plcR* genes of randomly selected *B. mycoides* isolates from rhizosphere, endosphere, bulk soil and microscale bulk soil samples to determine their pherotypes. We also analysed the diversity within these pherotypes and the distribution of the pherotypes between plants, their endosphere and rhizosphere, as well as their distribution across ERIC-types. To our knowledge this is a first such study on a species of *B. cereus* s. l. Isolates of *B. cereus* s. l. were, rather, isolated from different parts of the world and from different habitats (patient, animal, field etc.) and then analysed together (Ko et al., 2004; Lereclus et al., 1996; Slamti and Lereclus, 2002; Slamti and Lereclus, 2005). The quorum sensing system genes of our isolates clustered into groups PlcR-3 and PlcR-4. What is interesting is, that the bacteria isolated from rhizosphere of the same plant, or bacteria isolated from the same microscale soil samples, clustered into both groups, thereby showing their abilities to co-exist within very small distances. Our findings are consistent with the findings by Štefanič and Mandić Mulec (2009), who addressed polymorphism and distribution of *B. subtilis* ComQXPA quorum sensing systems in small soil samples and found coexistence of three pherotypes. Recently, Oślizło et al. (2015) have shown that in the tomato rhizosphere, three pherotypes of the *B. subtilis* ComQXPA quorum sensing system coexisted on roots of a single tomato plant, which is consistent with the results of our study. All of Miranda bulk soil isolates, sampled meter apart, were of PlcR-3 pherotype. The representatives of PlcR-4 pherotype were detected amongst soil samples only when microscale isolates were analysed, indicating higher diversity at the small scale than at the large scale, which supports the hypothesis that pherotype diversity is especially important or even advantageous at small scale where strains have the opportunity for interactions (Štefanič and Mandić Mulec, 2009). The Seresta bulk soil isolates had two representatives of the PlcR-4 pherotype, while the rest of the isolates belonged to the PlcR-3 pherotype. Interestingly, the *plcR* genes from bulk soil within both pherotypes showed 100% similarity, with the exception of SB-4. Such uniformity in bulk soil was not expected, especially when considering previous similar studies, done on *B. subtilis* (Štefanič and Mandić Mulec, 2009; Štefanič et al., 2012). The observed phenomenon contrasts the comparably higher diversity of *B. mycoides* observed at the level of ERIC-typing within the bulk soil population of the potato field. Based on our results from ERIC-PCR experiments and the conclusions of aforementioned studies on diversity of quorum sensing systems, we would expect to find more pherotypes (three or even four) and less similar gene sequences in the bulk soil. Knowing the effect plants can have on microbial populations, the high frequency of a single pherotype (PlcR-3 in our case) in the bulk soil population as compared to rhizosphere and endosphere populations could be caused by a change of conditions in the soil surrounding the roots, established by the presence of the potato plant. This single pherotype dominance could be established through a phenomenon known as facultative cheating. Here, a minority pherotype can co-exist, in low numbers, with a dominant pherotype and then increase its presence after changes in its environment,

caused by the plant, occurr (Eldar, 2011; Travisano and Velicer, 2004). That means that dominance would be stochastically determined. According to the "tragedy of the commons" theory, it might be advantageous for a pherotype to be less frequent, which may then result in continuous fluctuations of pherotype frequency (Štefanič et al., 2012). We also examined rhizosphere and endosphere separately from bulk soil isolates and observed equal distribution of PlcR-3 and PlcR-4 pherotypes between the two habitats as a whole, which suggests that the habitat itself does not select for a specific pherotype. However, upon comparison of the distribution of pherotypes between endosphere and rhizosphere of each separate plant, we observed that within plant no.1 and plant no.2 the dominant pherotype in the rhizosphere was not dominant in the endosphere. For example, in plant no.1 the dominant pherotype in the rhizosphere was PlcR-3 and in the endosphere PlcR-4. In plant no.3, however, the dominant pherotype (PlcR-3) in the endosphere was also dominant in rhizosphere. This would suggest that dominance of particular pherotype in the endosphere might be stochastically determined. Interestingly, when we analysed the correlation between ERIC-types and pherotypes the results indicated that most ERIC-type groups include only one of the two pherotypes, with the exception of three groups. This observation was only in part consistent with the study by Štefanič et al., 2012, who found diversity of pherotypes within housekeeping gene sequence clusters (or putative ecotypes) of highly related strains. In *B. subtilis*, each sequence cluster was dominated by a specific pherotype while other pherotypes were in minority. We cannot directly compare putative ecotype clusters to ERIC-type clusters, but the homogeneity of pherotype within most of the ERIC-type clusters suggested that the *plcR* genes of *B. mycoides* are less diverse than the *comQ* genes of *Bacillus subtilis*. It would be interesting to explore the role of *plcR* based quorum sensing system for survival in soil, or its role in plant-microbe interactions, and to research at which levels of entering into the plant's endosphere is the function of bacterial quorum sensing system most important. It would also be interesting to research and understand selective pressures that drive pherotype diversity of *B. mycoides* and of bacteria in general.

6 CONCLUSIONS

- *Bacillus mycoides* isolates from the potato endosphere clustered into several groups or ERIC-types, which is contrary to our hypothesis where we predicted that the endosphere would consist of a single ERIC-type.

- Based on ERIC-PCR typing and diversity indices we concluded that a lower diversity of *B. mycoides* isolates was associated with the potato endosphere rather than with the rhizosphere and bulk soil. This is consistent with our predictions and supports the hypothesis that the plant influences the diversity of *B. mycoides*. Similarly, the diversity of rhizosphere isolates was lower than that of bulk soil isolates, which also supports our hypothesis.

- The quorum sensing system gene *plcR* was found in the *B. mycoides* populations in bulk and rhizosphere soil and the endosphere. The sequences clustered into two of four known pherotypes.

- Our results showed that the diversity of *B. mycoides* pherotypes in the potato rhizosphere is comparable with the diversity of pherotypes in the endosphere, with both habitats containing the same pherotypes in equal proportions. Regarding the bulk soil isolates, however, we observed that one pherotype was present in higher frequency. These results do not support our hypothesis, which states that rhizosphere and bulk soil would show higher diversity of pherotypes than the endosphere.

- When analyzed separately, three potato plants showed different proportions of a particular *B. mycoides* pherotype in the endosphere or rhizosphere.

- In future, it would be interesting to determine the specific factors in plant's rhizosphere and at the level of plant-entry that exclude some strains of *B. mycoides* and determine the dominant presence of other. Likewise it would be interesting to determine the function of quorum sensing systems in this process and the pherotypes associated with them, as well as at which level this function has the highest importance.

7 SUMMARY

Microbial diversity in soil has an important functional role, as it can affect soil structure, nutrient cycling and soil water retention. It can affect plant growth, can cause disease or provide pest control (Pimentel et al., 1997). Plants in turn are known to release vast amounts of simple to highly complex root exudates, which include primary metabolites, sugars, secondary metabolites and enzymes (Bais et al., 2006; Bertin et al. 2003). They include other compounds as well, such as signal molecules, which enable cross kingdom communication with desirable organisms (Lugtenberg et al., 2002) and antimicrobial compounds called phytoalexins, which create a selective environment that can favour a sub-population of bacterial pool over others (Reva et al., 2004). These compounds create a selective environment, which attracts only a sub-population of the bacterial pool, while blocking others that reside in soil, and are plant's way of selecting and maintaining a desirable rhizosphere microflora (Bais et al., 2006). In a preliminary study, we recognized *B. mycoides* as a potential potato endophyte.

Our goal in this work was to analyse the diversity and structure of *B. mycoides* populations in rhizosphere and endosphere of potato and corresponding soil of garden bed using ERIC-PCR, a rep-PCR fingerprinting technique. We also analysed the diversity and distribution of the quorum sensing system pherotypes of *B. mycoides* in this environment, through analysis of *plcR-papR* gene fragments. We hypothesized that diversity of *B. mycoides* population in bulk soil will be greater than in rhizosphere, and that the latter will be greater than diversity of endosphere population. Furthermore, we hypothesized that endophytes will cluster into a single ERIC-type and that the diversity of the endophytes will be very low. We also analysed the diversity of the quorum sensing genes that determine the pherotypes in this environment. We hypothesized that bulk soil population will be characterised by several different pherotypes, but that endosphere population will contain only one pherotype.

We isolated 350 *B. mycoides* strains from endosphere and rhizosphere of three potato plants that belonged to cultivars Miranda and Seresta, and from bulk soil of the respective garden beds. The soil sampling points were one meter from each other, and the two garden beds – Miranda and Seresta were 15 m apart. In diversity analysis we found that there are three dominant OTUs (which we termed ERIC-types), each in its own endosphere. Such dominant types were not present in rhizosphere, where *B. mycoides* populations were more heterogenic than in endosphere. We reasoned that the dominance of a particular ERIC-type could be a consequence of a stochastic process, driven by the selective environment in plant's rhizosphere, created by root exudates. We calculated Shannon-Wiener index, a common diversity estimate. The number of strains isolated from bulk soil (25) was disproportional compared to rhizosphere and endosphere (60 per each habitat). For this reason, we performed random re-sampling of endosphere and rhizosphere isolates, with which we created sub-populations of 25 isolates. The process was repeated three times.

The results showed that the highest diversity index was that of bulk soil populations, followed by rhizosphere populations and endosphere populations.

We also analysed the diversity of gene fragments coding the PlcR-PapR quorum sensing system, which is a pleiotropic transcription regulator and is common to all species from the *B. cereus* s. l. clade. We were interested in its diversity, and distribution of pherotypes (quorum sensing types). We sequenced 114 *plcR* genes of randomly selected isolates. We selected equal number of isolates from all plant habitats (endosphere and rhizosphere) from all plants (plant 1, 2 and 3). We also selected an equal number of genes from microscale soil samples of MB-3 and MB-3 isolates. We compared the acquired sequences with reference sequences with determined pherotypes. The data obtained indicated the presence of two out of four known pherotypes within our collection of isolates. The PlcR-3 pherotype was the dominant and the PlcR-4 pherotype the minority pherotype in bulk soil. It was further observed only within the collection of isolates from microscale soil samples. In rhizosphere and endosphere the frequency of both pherotypes was comparable. However, when each plant was separately examined, one of the two pherotypes always dominated the given habitat. What is interesting is, that the bacteria isolated from rhizosphere of the same plant, or bacteria isolated from the same microscale soil samples, clustered into both groups, thereby showing their abilities to co-exist within very small distances, which was consistent with similar studies. We also observed a correlation between ERIC-types and pherotypes, which indicated that most ERIC-type groups include only one of the two pherotypes, with the exception of three groups.

7.1 POVZETEK

Mikrobna raznolikost je pomemben dejavnik, ki vpliva na funkcijo ekosistema ter na rast rastlin in je torej pomemben dejavnik tudi iz agronomskega vidika. Vpliv mikroorganizmov na rastline lahko sega od koristnega, kot je npr. spodbujanje rasti in zatiranje škodljivcev, do mutualističnega, ali pa antagonističnega, kjer so bakterije fitopatogene (Pimentel in sod., 1997). Tudi rastline s svojim delovanjem, preko sproščanja koreninskih izločkov sooblikujejo okolje okrog korenin in tako vplivajo na mikrobno združbo v tleh. Koreninski izločki vsebujejo primarne metabolite, sladkorje in sekundarne metabolite ter encime (Bais in sod., 2006; Bertin in sod., 2003). Vsebujejo tudi signalne molekule, ki rastlini omogočajo komuniciranje z mikroorganizmi iz drugih kraljestev v zemlji (Lugtenberg in sod., 2004) in protimikrobne snovi imenovane fitoaleksini, ki v okolju ustvarijo selektivni pritisk (Reva in sod., 2004) in na ta način ohranjajo koristno bakterijsko populacijo v svojem okolju (Bais in sod., 2006). Naš cilj je bil oceniti genetsko raznolikost bakterij vrste *Bacillus mycoides,* ki smo jih osamili iz tal krompirjevih polj ter iz rizosfere in endosfere krompirja serovarov Miranda in Seresta. Polje, kjer smo tla vzorčili se nahaja v mestu Eursinge, v občini Midden-Drenthe, provinci Drenthe na Nizozemskem. Pri izolatih *B. mycoides* smo poleg genomske raznolikosti analizirali tudi

raznolikost in distribucijo sistemov zaznavanja celične gostote (kvoruma) oz. raznolikost fragmentov lokusa, ki nosi zapis za ta sistem.

Vrsta *Bacillus mycoides* sodi v taksonomsko skupino *Bacillu cereus* sensu lato (s. l.), ki zajema sedem genetsko zelo sorodnih bakterijskih vrst: *B. cereus* sensu stricto (s. s.), *B. thuringiensis*, *B. antracis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides* in *B. cytotoxicus* (Ceuppens in sod., 2013). Vrste znotraj taksonomske skupine *B. cereus* s. l. so si, kljub velikimi fenotipskimi razlikami, kot je virulenca, temperaturni optimum, morfologija ipd., med seboj filogenetsko izredno sorodne (Carlson in sod., 1994; Helgason in sod., 2004; Ticknor in sod., 2001), kar se izraža tako v nukleotidni sestavi njihovih genomov (DNA-DNA hibridizacija) (Kaneko in sod., 1978) kakor tudi v podobnosti 16 S rRNA in 23 S rRNA genov, posledično pa tovrstne analize ne zagotavljajo jasne ločitve vrst na podlagi genetskih razlik. (Ash in sod., 1991; Ash in Collins, 1992). Tudi poskusi ločevanja *B. cereus* s. s. in *B. thuringiensis* z uporabo kompleksnejših metod, kot so MLEE, REDP in PFGE (Helgason et al., 2000; Helgason et al. 2004) ali celo kombinacija PFGE in MLEE ni omogočila jasnega razlikovanja med vrstama (Carlson in sod., 1994). Za zdaj je seve vrste *B. cereus* s. l. in *B. thuringiensis* mogoče jasno ločiti le na osnovi plazmida na katerem se nahaja zapis za *cry* insekticidne proteine in je značilen za *B. thuringensis*. V kolikor bakterija ta plazmid izgubi, je praktično neločljiva od *B. cereus* s. s., kar kaže na problematičnost ločevanja vrst te taksonomske skupine na podlagi genetike. *B. mycoides* je po Gramu pozitivna, negibljiva bakterija, ki se nahaja pretežno v tleh in tvori endospore (Di Franco in sod., 2002). Na to vrsto smo se osredotočili, ker so preliminarni rezultati pokazali, da je lahko potencialni endofit krompirja (Spoelder in sod., neobjavljeni podatki), kar v znanstveni literaturi še ni bilo predhodno objavljeno, vendar pa so to vrsto že opisali kot potencialni endofit in biokontrolni agens pri drugih rastlinah (Paul in sod., 1995; Bargabus in sod., 2002). Na trdnih gojiščih ima unikatno, glivam podobno morfologijo kolonij, ki se razraščajo bodisi v smeri urinega kazalca ali pa v nasprotni smeri. Na morfologijo kolonij vplivajo številni fizikalno-kemijski parametri: npr. temperatura, ki nad 35 °C povzroči krajšo rast filamentov, nad 37 °C pa izgubo unikatne morfologije (Di Franco in sod., 2002).

Pri bakterijah je znana medcelična komunikacija s sistemi zaznavanja celične gostote oz. kvoruma (Miller in Bassler, 2001). Pripadniki skupine *B. cereus* s. l. se sporazumevajo s sistemom PlcR-PapR (PlcR je receptor in PapR signal), katerega zapis nosi lokus *plcR-papR*. Na podlagi analize strukture PlcR proteina so le-tega uvrstili v družino RNPP regulatorjev. PlcR je poglavitni transkripcijski regulator številnih genov in regulira tudi lastno izražanje, oz. izražanje gena *plcR* (Lereclus in sod., 1996; Slamti in Lereclus, 2002). Pri vrstah *B. cereus* s. s. in *B. thuringiensis* so odkrili, da nadzira prepisovanje več genov, ki so potencialno povezani z virulenco (Bouillaut in sod., 2008; Gohar in sod., 2008; Lereclus in sod., Slamti in Lereclus, 2002). Za sisteme zaznavanja kvoruma pa je značilen polimorfizem oz., razdelitev na več ferotipov, za katere velja specifičnost prepoznavanja signala (Bouillaut in sod., 2008; Slamti in Lereclus, 2005; Štefanič in Mandič Mulec,

2009). V primeru PlcR-PapR to pomeni razdelitev v štiri različne ferotipske skupine: PlcR skupina I, PlcR skupina II, PlcR skupina III in PlcR skupina IV (Lereclus in sod., 1996; Slamti in Lereclus, 2002). (slednje smo za okvir te naloge skrajšali v ferotip PlcR-1, PlcR-2, PlcR-3 in PlcR-4).

V okviru te magistrske naloge smo osamili skupno 350 izolatov vste *B. mycoides* iz tal s krompirjem zasajenega polja, ter iz rizosfere in endosfere dveh kultivarjev krompirja: Miranda in Seresta. Vzorci tal so bili med seboj razmaknjeni po en meter, obe gredi, Miranda in Seresta, pa sta bili druga od druge oddaljeni 15 metrov. Vzorce tal smo suspendirali v fiziološki raztopini in jih toplotno obdelali pri temperaturi 80 °C, 15 minut. Slednji korak uniči vse vegetativne celice in ohrani spore, kar drastično zmanjša število kolonij na agarskih gojiščih in poenostavi osamitev želenih bakterij. Vzorce rizosfere smo pridobili tako, da smo koreninice krompirja s tankim slojem zemlje spirali v PBS pufru z dodanimi steklenimi kroglicami. Po končanem spiranju smo koreninice odstranili, suspenzijo pa toplotno obdelali enako kot vzorce tal. Pridobljene spore smo nacepili na hranilni agar, ki smo ga inkubirali 48 ur pri 28 °C. Izolate iz endosfere smo pridobili iz koreninic, ki so ostale pri pridobivanju vzorcev rizosfere. Koreninice smo površinsko sterilizirali s 70 % etanolom in jih temeljito sprali z deionizirano vodo. Nato smo jih s sterilnim skalpelom razrezali na izredno majhne koščke in jih položili na hranilni agar, ki smo ga inkubirali 48 ur pri 28°C. Bakterije smo selekcionirali na podlagi njihove edinstvene, lahko prepoznavne morfologije. Vse kolonije, ki so ustrezale morfologiji *B. mycoides* smo postrgali s sterilnimi zobotrebci in jih nacepili na svež hranilni agar. Plošče smo inkubirali 48 ur pri temperaturi 28 °C. V nadaljevanju smo s cepilno zanko kolonije trikrat precepili na sveže gojišče in s tem pridobili čisto kulturo. Pridobljene izolate smo shranili v obliki tekoče kulture z dodanim glicerolom (25 %) pri -70 °C. Izolate smo tipizirali z metodo ERIC-PCR. Metoda temelji na pomnoževanju DNA z oligonukleotidnimi začetniki, ki nalegajo na zaporedja, razpršena po celotnem genomu bakterije, ki pa so ohranjena v različnih rodovih bakterij, odkrili pa so jih celo v evkariontskih ter virusnih genomih (Gillings in Holley, 1997; Versalovic in sod., 1991). Na tem mestu je potrebno pripomniti, da smo v našem primeru uporabili nižjo temperaturo naleganja, kot je predvideno v protokolu avtorjev Versalovic in sod. (1994) in sicer 45 °C namesto 52 °C. Za potrditev specifičnosti in konsistence reakcije smo izvedli test, kjer smo analizirali tri naključno izbrane izolate, vsakega v šestih neodvisno pripravljenih PCR mešanicah po protokolu s spremenjeno, nižjo temperaturo naleganja. Test je potrdil ponovljivost PCR reakcije izvedene po protokolu s spremenjeno temperaturo naleganja.

Pridobljene PCR reakcijske mešanice smo analizirali z elektroforezo v agaroznem gelu in tako za vsak sev pridobili prstni odtis (vzorec lis). Slike elektroforetskih lis smo analizirali s programom BioNumerics (Applied Maths), ki označene elektroforetske lise pretvori v binarno matrico prisotnosti in odsotnosti lis. Na podlagi te matrice smo z uporabo algoritma (v našem primeru Jaccard) izračunali matrico podobnosti, ter na podlagi slednje izrisali dendrogram podobnosti. Za statistično oceno raznolikosti populacij *B. mycoides* v

tleh, endosferi in rizosferi krompirja, smo uporabili Shannon-Wienerjev indeks, ki je pogosto rabljen za ugotavljanje raznolikosti pri ekoloških raziskavah (Ge in sod., 2008).

V okviru te magistrske naloge smo preučevali tudi PlcR-PapR sistem za zaznavanje celične gostote pri izolatih *B. mycoides*. Pri taksonomski skupini *B. cereus* s. l. je ta sistem izredno raznolik, geni *plcR* pa se združujejo v štiri podobnostne skupine, ki sovpadajo s komunikacijskimi skupinami oz. ferotipi (Slamti in Lereclus, 2005). Zanimala nas je predvsem porazdelitev izolatov *B. mycoides* v različne ferotipe. V ta namen smo pomnožili in sekvencirali gen *plcR*, (gen za ferotipe PlcR-1, PlcR-2 in PlcR-3 je velik 858 bp, gen za ferotip PlcR-4 pa 864 bp) 114 naključno izbranih izolatov *B. mycoides* iz naše zbirke. Iz vsakega okolja (endosfera, rizosfera) treh rastlin kultivarja Miranda smo izbrali enako število izolatov (10). Poleg tega pa smo v anlizo vključili tudi izolate gred obeh kultivarjev krompirja (13 na gredo) ter iz mikrookolji MB-3 in MB-13 (14 na mikrookolje). Pridobljene sekvence smo analizirali skupaj s sekvencami dostopnimi v podatkovnih bazah, katerih ferotipi so bili predhodno že določeni (Slamti in Lereclus, 2002; Slamti in Lereclus, 2005).

Skupno smo v okviru te magistrske naloge izolirali 350 izolatov. Iz vsake grede smo pridobili po 13 vzorcev tal, skupno torej 26 vzorcev iz katerih smo pridobili po en izolat. Poleg tega smo iz vzorcev tal št. 3, 8 in 13 obeh kultivarjev krompirja izolirali še dodatnih 14 sevov na vzorec. Ti so predstavljali populacije iz mikrookolij, saj je vseh 14 izolatov izhajalo iz istega grama vzorca tal. Iz rizosfere in endosfere smo osamili po 20 izolatov in sicer smo bakterije izolirali iz treh rastlin obeh kultivarjev, tako smo pridobili 60 izolatov iz rizosfer in 60 izolatov iz endosfer obeh kultivarjev krompirja. Pri analizi raznolikosti z metodo ERIC-PCR, smo se omejili na izolate kultivarja Miranda in izolate iz zemlje grede Seresta. V primeru enega od izolatov iz zemlje (izolat MB-3) je bila PCR reakcija neuspešna, zato izolata pri analizi nismo uporabili, posledično je bilo torej v analizo vključenih 25 izolatov iz zemlje namesto 26. Število analiziranih izolatov iz tal (skupaj 25) je bilo v primerjavi s številom izolatov iz rizosfere in endosfere (skupaj 120) neenakovredno, zaradi česar smo z računalniško vodenim naključnim vzorčenjem izolatov iz nabora iz rizosfere in endosfere izbrali po 25 izolatov. Ta korak smo izvedli trikrat. Najvišjo vrednost Shannon-Wienerjevega indeksa smo izračunali za populacijo tal (H = 2.32), indeks populacije izolirane iz rizosfere je bil nižji (povprečje H = 2.02 ± 0,18) in indeks populacije endosfere je bil najnižji (povprečje H = 1,50 ± 0,22). Rezultati, ki smo jih pridobili, so skladni z našo hipotezo, da se raznolikost *B. mycoides* znižuje v smeri tla, rizosfera, endosfera. Med izolati iz endosfere smo zaznali tri prevladujoče ERIC-tipe, pri čemer je v vsaki posamezni rastlini prevladal le eden. V rizosferi je bila raznolikost ERIC-tipov večja in prevlade samo enega tipa nismo zaznali. Kot smo že v uvodu omenili, rastlina z izločanjem kompleksnih koreninskih izločkov v svoji rizosferi ustvari selektivno okolje. Ta dejavnik verjetno lahko pojasni upad raznolikosti populacije *B. mycoides* v rizosferi, v primerjavi z raznolikostjo v tleh. Poleg tega je zmožnost bakterij, da kolonizirajo površino korenine ena od ključnih lastnosti, ki jim omogoča vstop v endosfero

rastline, kar pa lahko pojasni zmanjšano raznolikost populacije endofitov v primerjavi z rizosferno populacijo *B. mycoides*. Na tem mestu je potrebno pripomniti, da ne moremo zagotoviti, da so izolati iz endosfere dejansko endofiti, saj so spore vrst *Bacillus* odporne na sterilizacijo z etanolom (Thomas, 2012) in je povsem mogoče, da pridobljeni izolati izvirajo iz spor bakterij, ki so kolonizirale površino korenin.

Poleg raznolikosti med sevi, smo analizirali tudi 101 gen *plcR* (kvaliteta preostanka sekvenc ni bila zadovoljiva), ki kodira receptor sistema zaznavanja kvoruma. Sekvence *plcR* genov naših izolatov smo primerjali z referenčnimi geni iz vseh štirih ferotipov. V tleh je bil ferotip PlcR-3 prevladujoč, ferotip PlcR-4 pa smo zaznali šele med izolati, ki so izhajali iz mikrookolji v tleh. Porazdelitev obeh ferotipov med endosferami in rizosferami vseh rastlin je bila enakomerna. Opazili smo, da je bilo število predstavnikov ferotipa PlcR-4 višje v/na rastlini kot v tleh, kar nakazuje odzivnost tega ferotipa na okolje, ki ga ustvari rastlina. V podobnih raziskavah raznolikost ferotipov sistema ComQXPA pri vrstah *Bacillus* v zemlji (Štefanič in Mandić Mulec, 2009) in koreninski površini paradižnika (Oślizło in sod., 2015) so odkrili večjo raznolikost ferotipov, in sicer od tri do štiri ferotipe. Vseeno pa je prisotnost dveh ferotipov v rizosferi, endosferi in v mikrookolju tal skladna s predvidevanjem, da je raznolikost ferotipov na majhnih razdaljah koristna za obstoj vrst (Štefanič in sod., 2012). oziroma, da je raznolikost adaptivna (Eldar, 2011; Travisano in Velicer, 2004). Naša raziskava je prva, ki pokaže na raznolikost ferotipov v naravnih populacijah *B. mycoides* v odvisnosti od habitata (tla:rastlina).

Združitev rezultatov ERIC-PCR analize ter analize sistema zaznavanja kvoruma je pokazala, da je večinoma en ERIC-tip zastopan samo z enim od obeh ferotipov (PlcR-3 in PlcR-4) in le trije ERIC-tipi zastopani z obema ferotipoma, kar kaže na zelo enotno porazdelitev ferotipov med genetsko podobnimi *B. mycoides*, v primerjavi s podobnimi raziskavami opravljenimi na *B. subtilis*. V prihodnje bi bilo zanimivo raziskati, kateri dejavniki v rizosferi in na stopnji vstopa bakterije v rastlino, zavirajo rast nekaterih sevov *B. mycoides* omogočajo pa rast in kolonizacijo drugih in kakšno vlogo pri tem imajo sistemi zaznavanja kvoruma oz. z njimi povezani ferotipi.

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ANNEXES

Annex A: ERIC-PCR dendrograms for diversity index calculations. The following pages include dendrograms constructed based on randomly selected isolates from rhizosphere and endosphere, as well as dendrogram based on fingerprints of all bulk soil isolates. This page includes information about symbols used in the following calculations.

Priloga A: ERIC-PCR dendrogrami za izračun indeksov raznolikosti. Naslednje strani vsebujejo dendrograme izrisane na podlagi naključno izbranih izolatov iz rizosfere in endosfere, kot tudi dendrograme na podlagi genetskih prstnih odtisov (vzorcev lis) izolatov iz vzorcev tal. Ta stran vsebuje informacije o simbolih ki smo jih uporabili v izračunih raznolikosti.

OTU = Operational taxonomic unit

n_i = number of representatives of an ERIC type

N = number of OTUs

P_i = proportion of ERIC types

ln = natural logarithm

n = total number of strains

H’ = Shannon-Wiener index

OTU = Operacijska taksonomska enota

n_i = število predstavnikov določenega ERIC-tipa

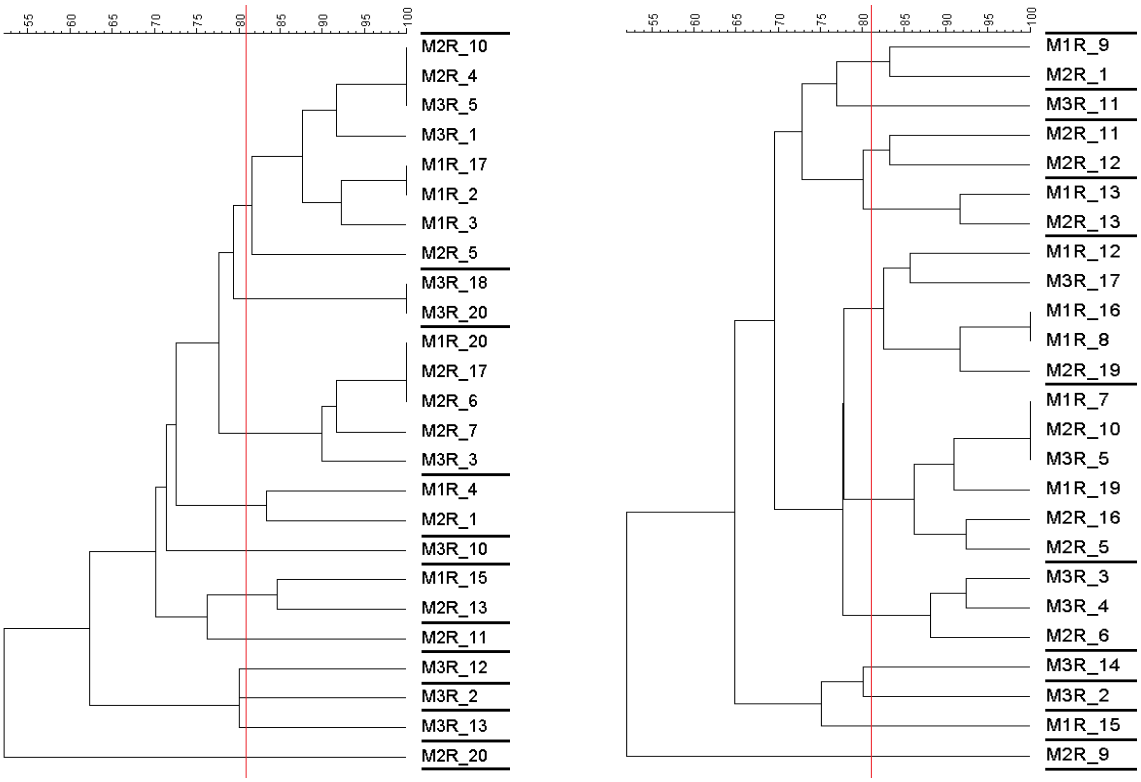
N = število OTU

P_i = delež ERIC-tipov

ln = naravni logaritem

n = število vseh izolatov

H’ = Shannon-Wienerjev indeks



Annex A1: Similarity dendrograms based on randomly selected fingerprints from individual habitats. Left: Sample of **rhizosphere** isolates no.1; Right: Sample of **rhizosphere** isolates no.2. Vertical red line represents 81% similarity cut-off. OTUs are separated with horizontal black line.
Priloga A1: Dendrograma podobnosti, ki temeljita na naključno izbranih vzorcih lis iz posameznih habitatov. Levo: Vzorec **rizosfernih** izolatov št. 1; Desno: Vzorec **rizosfernih** izolatov št. 2. Navpična rdeča črta predstavlja mejo 81% podobnosti. OTU so ločene s horizontalnimi črnimi črtami.

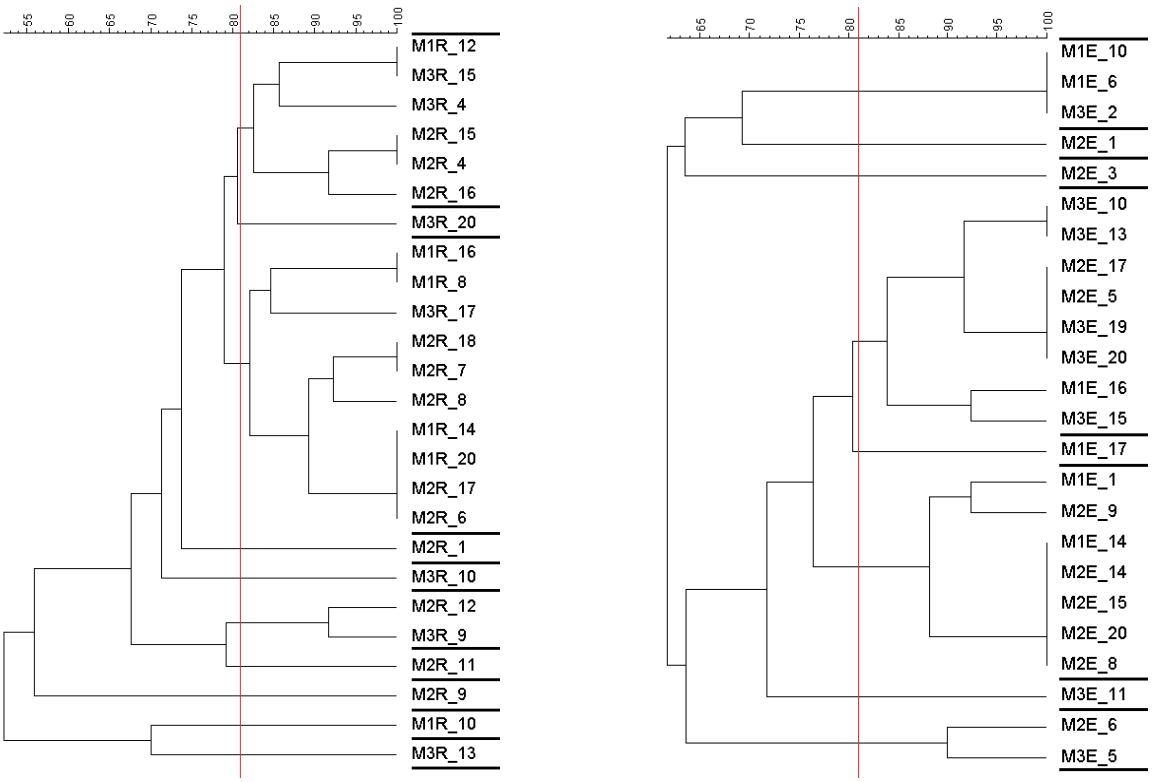
Annex A2: Table of Shannon-Wiener index calculation: Left: rhizosphere sample no. 1; Right: rhizosphere sample no. 2.
Priloga A2: Preglednica izračuna Shannon-Wienerjevega indeksa: Levo: rizosferni vzorec št. 1; Desno: rizosferni vzorec št. 2.

OTU	n_i	P*i*	ln P*i*	P*i*(lnP*i*)
A	8	0.32	-1.1394	-0.3646
B	2	0.08	-2.5257	-0.2021
C	5	0.20	-1.6094	-0.3219
D	2	0.08	-2.5257	-0.2021
E	1	0.04	-3.2189	-0.1288
F	2	0.08	-2.5257	-0.2021
G	1	0.04	-3.2189	-0.1288
H	1	0.04	-3.2189	-0.1288
I	1	0.04	-3.2189	-0.1288
J	1	0.04	-3.2189	-0.1288
K	1	0.04	-3.2189	-0.1288
				= -2.0652

N =	11
n =	25
H' =	2.0652

OTU	n_i	P*i*	ln P*i*	P*i*(lnP*i*)
A	2	0.08	-2.5257	-0.2021
B	1	0.04	-3.2189	-0.1288
C	2	0.08	-2.5257	-0.2021
D	2	0.08	-2.5257	-0.2021
E	5	0.20	-1.6094	-0.3219
F	6	0.24	-1.4271	-0.3425
G	3	0.12	-2.1203	-0.2544
H	1	0.04	-3.2189	-0.1288
I	1	0.04	-3.2189	-0.1288
J	1	0.04	-3.2189	-0.1288
K	1	0.04	-3.2189	-0.1288
				= -2.1688

N =	11
n =	25
H' =	2.1688



Annex A3: Similarity dendrograms based on randomly selected fingerprints from individual habitats. Left: Sample of **rhizosphere** isolates no. 3; Right: Sample of **endosphere** isolates no. 1. Red line represents 81% similarity cut-off. OTUs are separated with horizontal black line.
Priloga A3: Dendrograma podobnosti, ki temeljita na naključno izbranih vzorcih lis iz posameznih habitatov. Levo: Vzorec **rizosfernih** izolatov št. 3; Desno: Vzorec **endosfernih** izolatov št. 1. Navpična rdeča črta predstavlja mejo 81% podobnosti. OTU so ločene s horizontalnimi črnimi črtami.

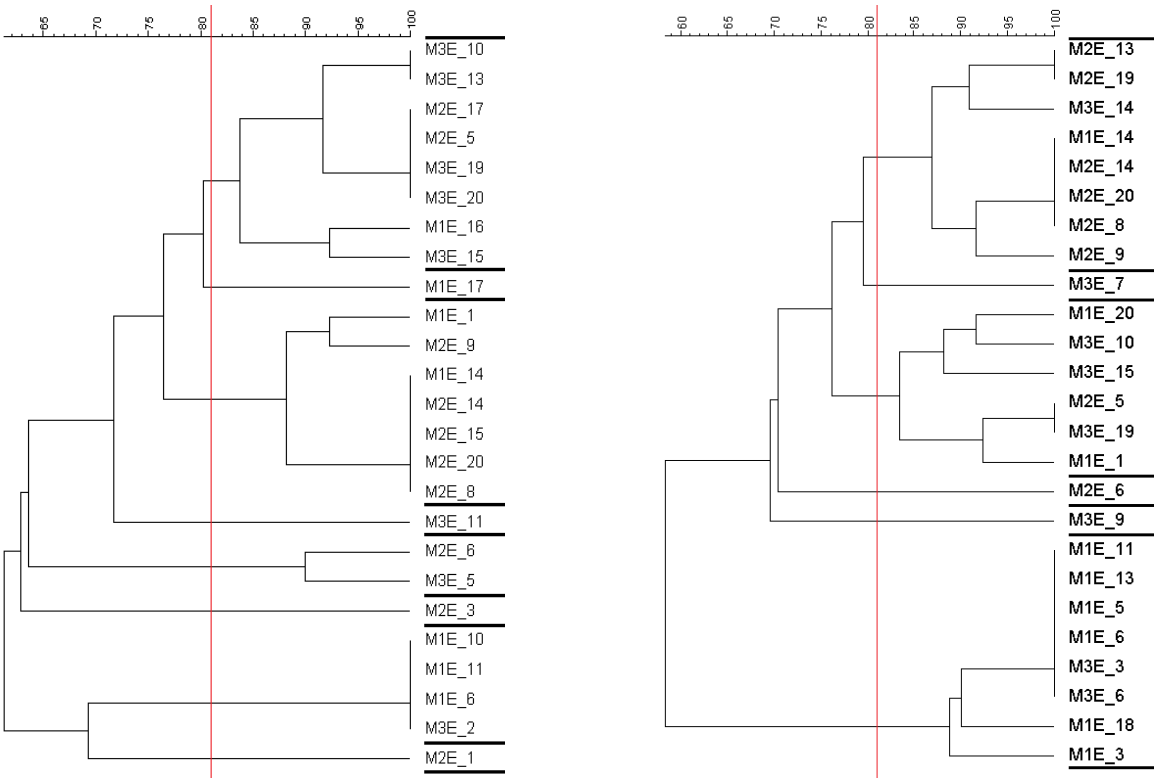
Annex A4: Table of Shannon-Wiener index calculation: Left: rhizosphere sample no. 3; Right: endosphere sample no. 1.
Priloga A4: Preglednica izračuna Shannon-Wienerjevega indeksa: Levo: rizosferni vzorec št. 3; Desno: endosferni vzorec št. 1.

OTU	n_i	Pi	ln Pi	Pi(lnPi)
A	6	0.24	-1.4271	-0.3425
B	1	0.04	-3.2189	-0.1288
C	10	0.40	-0.9163	-0.3665
D	1	0.04	-3.2189	-0.1288
E	1	0.04	-3.2189	-0.1288
F	2	0.08	-2.5257	-0.2021
G	1	0.04	-3.2189	-0.1288
H	1	0.04	-3.2189	-0.1288
I	1	0.04	-3.2189	-0.1288
J	1	0.04	-3.2189	-0.1288
				= -1.8124

N =	10
n =	25
H' =	1.8124

OTU	n_i	Pi	ln Pi	Pi(lnPi)
A	8	0.32	-1.1394	-0.3646
B	1	0.04	-3.2189	-0.1288
C	6	0.24	-1.4271	-0.3425
D	1	0.04	-3.2189	-0.1288
E	1	0.04	-3.2189	-0.1288
F	8	0.32	-1.1394	-0.3646
				= -1.4580

N =	6
n =	25
H' =	1.4580



Annex A5: Similarity dendrograms based on randomly selected fingerprints from individual habitats. Left: Sample of **endosphere** isolates no. 2; Right: Sample of **endosphere** isolates no. 3. Red line represents 81% similarity cut-off. OTUs are separated with horizontal black line.
Priloga A5: Dendrograma podobnosti, ki temeljita na naključno izbranih vzorcih lis iz posameznih habitatov. Levo: Vzorec **endosfernih** izolatov št. 2; Desno: Vzorec **endosfernih** izolatov št. 3. Navpična rdeča črta predstavlja mejo 81% podobnosti. OTU so ločene s horizontalnimi črnimi črtami.

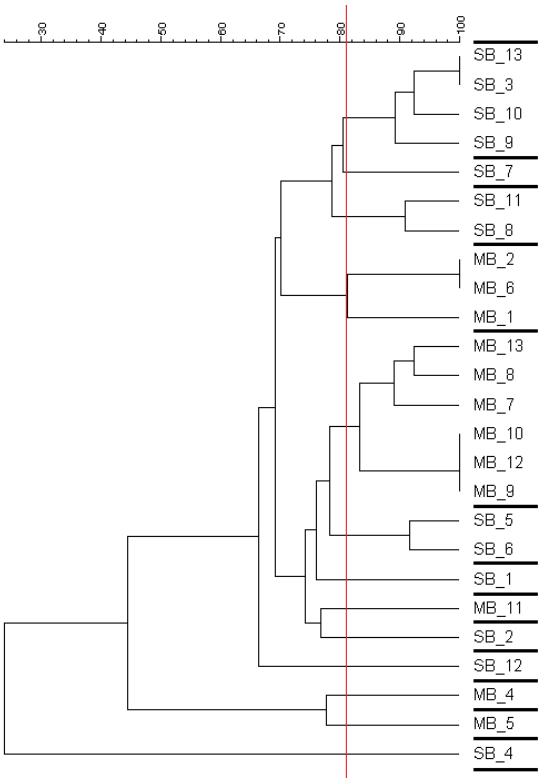
Annex A6: Table of Shannon-Wiener index calclation: Left: endosphere sample no. 2; Right: endosphere sample no. 3.
Priloga A6: Preglednica izračuna Shannon-Wienerjevega indeksa: Levo: endosferni vzorec št. 2; Desno: endosferni vzorec št. 3.

OTU	n_i	Pi	ln Pi	Pi(lnPi)
A	4	0.16	-1.8326	-0.2932
B	1	0.04	-3.2189	-0.1288
C	1	0.04	-3.2189	-0.1288
D	8	0.32	-1.1394	-0.3646
E	1	0.04	-3.2189	-0.1288
F	7	0.28	-1.2730	-0.3564
G	1	0.04	-3.2189	-0.1288
H	2	0.08	-2.5257	-0.2021
				= -1.7313

N =	12
n =	25
H' =	1.7313

OTU	n_i	Pi	ln Pi	Pi(lnPi)
A	7	0.28	-1.2730	-0.3564
B	1	0.04	-3.2189	-0.1288
C	11	0.44	-0.8210	-0.3612
D	1	0.04	-3.2189	-0.1288
E	5	0.20	-1.6094	-0.3219
				= -1.2971

N =	11
n =	25
H' =	1.2971



Annex A7: Similarity dendrogram based on fingerprints of the **bulk soil** isolates. Red line represents 81% similarity cut-off. OTUs are separated with horizontal black line.
Priloga A7: Dendrogram podobnost, ki temelji na vzorcih lis izolatov iz **tal**. Navpična rdeča črta predstavlja mejo 81% podobnosti. OTU so ločene s horizontalnimi črnimi črtami.

Annex A8: Table of Shannon-Wiener index calculation for bulk soil isolates.
Priloga A8: Preglednica izračuna Shannon-Wienerjevega indeksa za isolate iz tal

OTU	n_i	Pi	ln Pi	Pi (lnPi)
A	4	0.16	-1.83258	-0.29321
B	1	0.04	-3.21888	-0.12876
C	2	0.08	-2.52573	-0.20206
D	3	0.12	-2.12026	-0.25443
E	6	0.24	-1.42712	-0.34251
F	2	0.08	-2.52573	-0.20206
G	1	0.04	-3.21888	-0.12876
H	1	0.04	-3.21888	-0.12876
I	1	0.04	-3.21888	-0.12876
J	1	0.04	-3.21888	-0.12876
K	1	0.04	-3.21888	-0.12876
L	1	0.04	-3.21888	-0.12876
M	1	0.04	-3.21888	-0.12876
				-2.32431

N =	25
n =	13
H '=	2.324309431

Annex B: ERIC-PCR dendrogram of analysed isolates along with *B. mycoides* type strain. This shows how *B. mycoides* Flügge (type strain) clusters together with our isolates. The arrow marks the type strain. The dendogram is presented on the next page.

Priloga B: ERIC-PCR dendrogram analiziranih izolatov, skupaj z *B. mycoides* tipskim sevom. Dendrogram prikaže kako se *B. mycoides* Flügge (tip sev) uvršča z našimi izolati. Puščica označuje tipski sev. Dendogram je natisnjen na naslednji strani.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M1R-15
M2R-16
M2R-5
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M2E-4
M3E-7
M2R-18
M2R-7
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M1R-10
M2R-20
M2R-9
MB-4
MB-5
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Annex C: *plcR* sequences of our isolates.
Priloga C: *plcR* sekvence naših izolatov.

>M1E-3

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>SB-1

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>SB-2

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>SB-3

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>SB-4

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>SB-5

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