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Signature ……..

Characterisation of the *Pectobacterium aquaticum* isolated from Polish water in addition to studying the selected virulence and environmental factors important for development of potato blackleg and soft rot diseases

Charakterystyka szczepu *Pectobacterium aquaticum* wyizolowanego z polskich wód oraz zbadanie wybranych czynników bakteryjnych i środowiskowych istotnych dla rozwoju czarnej nóżki i mokrej zgnilizny na ziemniaku

Thesis submitted to the Board of the Discipline of Biotechnology of the University of Gdańsk in order to be awarded a doctoral degree in the field of exact and natural sciences in the discipline of biotechnological sciences

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1. Abstract

Pectinolytic bacteria from the *Dickeya* and *Pectobacterium* genera belonging to the Soft Rot *Pectobacteriaceae* (SRP) cause substantial damage associated with soft rot in crops, vegetables, and ornamental plants, as well as blackleg in potatoes. *Dickeya* spp. and *Pectobacterium* spp. rank among the top ten most important plant pathogenic bacteria globally due to the huge losses they cause in potato production. Despite the considerable financial impact and the prevalent occurrence of pectinolytic bacteria, there are currently no efficient plant protection strategies available to combat these pathogens. Until now, screening of potato tubers for latent infections with *Dickeya* spp. and *Pectobacterium* spp. has been regarded as the most effective method for protecting this crop. Therefore, the aims of this thesis were set as follows: i) assessment whether Polish waterways may be the source of bacteria from the family *Pectobacteriaceae*, which can cause symptoms of soft rot and blackleg diseases on potato, ii) the search for virulence determinants in *Pectobacterium aquaticum* and *Dickeya solani*, iii) the application of metagenomics for elucidation of the differences in the structure of bacterial microbiota in suppressive and non-suppressive soils in relation to Soft Rot *Pectobacteriaceae*.

Pectobacterium spp. and *Dickeya* spp. are present in various environmental habitats, including water. In order to determine the abundance of SRP in Pomeranian lakes, water samples were collected from different depths during two years monitoring period. Subsequent isolation and comprehensive genotypic and phenotypic characterisation revealed the presence of bacteria from the family *Pectobacteriaceae* in the studied lakes. Interestingly, during this research, an isolate belonging to the recently established species, *Pectobacterium aquaticum* was acquired. The identified and characterized *P. aquaticum* IFB5637 strain exhibited the production of plant cell wall degrading enzymes (PCWDEs), including pectinases, cellulases and proteases. Moreover, it was able to macerate potato and chicory tissues under laboratory conditions. This data indicates that utilizing of surface waters for the irrigation of potato fields may lead to dissemination of pectinolytic bacteria and the consequent appearance of the symptoms of soft rot and blackleg diseases.

The next part of the study was devoted to the identification of the virulence factors of *P. aquaticum* and *Dickeya solani*. Among virulence factors of plant pathogenic bacteria there are lipopolysaccharides (LPS) which play an important role in the initial phase of host-pathogen recognition. Therefore, subsequent research was focused on elucidating the chemical composition of the O-polysaccharide (OPS) of the *P. aquaticum* strain IFB5637 LPS. The analysis that was performed indicated that the structure of the OPS consists of common sugars like mannose and glucose, along with abequose. Notably, the latter one was a newly discovered constituent of O-antigen among the members of the *Pectobacteriaceae* family.

The ability of *D. solani* to cause severe diseases is attributed to the effective production of PCWDEs that break down the plant cell wall. Despite high genetic homogeneity of *D. solani* strains, some of them indicated extremely different virulence levels. Because there is still a lack of

understanding of the principles behind the noted differences in the pathogenicity of *D. solani*, our study included investigation of the methylomes and transcriptomes of two *D. solani* strains: the highly virulent IFB0099 and the low virulent IFB0223. Examination of the DNA methylation patterns showed no noticeable differences between the profiles of the analysed strains. However, RNA-Seq analysis revealed differences between the compared *D. solani* strains. Some of the genes encoding components of the secretion system II (T2SS) (*gspJ, nipE*) and three endo-pectate lyases (*pelD, pelE, pelL*) exhibited higher stimulation of expression under induced conditions in strain IFB0099 compared to IFB0223, which may lead to an increased virulence. Additionally, upregulation of genes encoding the proteins building up flagella in IFB0099 (*flgC, flgB, flgD, fliG, flgG, flgF, flhA, fliA*) in contrast to IFB0223 might result in increased mobility of the highly virulent strain.

The severity of disease caused by plant pathogenic bacteria depends on plant the host resistance, the bacterial virulence and environmental conditions. Therefore, it was examined whether the development of disease symptoms caused by *Dickeya* spp. and *Pectobacterium* spp. could be linked to the specific features of the soil, in particular the composition of the bacterial soil microbiota. According to the literature, the term ‘suppressive soil’ is used to describe soil in which the growing plants show no or minimal disease symptoms, even in the presence of a virulent pathogen. In contrast to the ‘suppressive soil’, a ‘non-suppressive soil’ is described as soil, whose microbiological composition favours development of disease symptoms. On the basis of an earlier monitoring of the occurrence of soft rot and blackleg on potato plantations, suppressive and non-suppressive soils towards the above-mentioned diseases were collected from two different locations. The performed analyses showed that suppressive and non-suppressive soils exhibited similar physical and chemical properties. However, 16S rRNA gene amplicon sequencing demonstrated differences in the composition of the bacterial microbiota between the two analysed soil types, *i.e.* representatives of the genera *Bacillus, Acidobacterium*, and *Gaiella* turned out to be much more abundant in the studied suppressive in contrast to the non-suppressive soil.

In summary, the performed research enriches knowledge on the complexity of interactions between economically significant plant pathogens from the genera *Dickeya* and *Pectobacterium*, the environment and host plants. It open a future perspective for better disease management and enhancing crop protection strategies. Though, further studies and advancements in understanding the virulence mechanisms and interactions involving SRP are essential for the development of efficient control measures and, by these means, boosting the sustainability of agricultural systems.

2. Streszczenie

Bakterie pektynolityczne z rodzajów *Dickeya* i *Pectobacterium*, należących do Soft Rot *Pectobacteriaceae* (SRP), powodują znaczne straty w plonach wywoływane przez mokrą zgniliznę ziemniaka, licznych warzyw i roślin ozdobnych, jak również czarną nóżkę ziemniaka. *Dickeya* spp. i *Pectobacterium* spp. zaliczane są do dziesięciu rodzajów bakteryjnych patogenów roślin, które powodują, największe w skali świata, straty w produkcji ziemniaka. Pomimo odnotowywanych wysokich strat ekonomicznych i powszechnego występowania bakterii pektynolitycznych w środowisku, nie opracowano jak dotąd skutecznej metody ochrony roślin przed tymi patogenami. Badanie bulw ziemniaka pod kątem obecności bakterii z rodzajów *Dickeya* spp. i *Pectobacterium* spp. i ich eliminacja z uprawy jest uważana za najskuteczniejszy sposób ochrony plonów przed tymi patogenami. W związku z przytoczonymi faktami cele przedmiotowej rozprawy doktorskiej zostało sformułowane następująco: i) stwierdzenie, czy naturalne zbiorniki wodne stanowią potencjalne źródło występowania bakterii z rodziny *Pectobacteriaceae*, patogenów które mogą wywoływać objawy mokrej zgnilizny i czarnej nóżki na ziemniaku, ii) poszukiwanie czynników wirulencji wpływających na patogeniczność bakterii z gatunków *Pectobacterium aquaticum* i *Dickeya solani*, iii) wykorzystanie podejścia metagenomicznego w celu zrozumienia różnic w strukturze mikrobioty bakteryjnej w glebie supresyjnej i niesupresyjnej wobec bakterii z rodziny *Pectobacteriaceae*.

Pectobacterium spp. i *Dickeya* spp. występują w różnych środowiskach, włączając w to zbiorniki wodne. W celu zbadania obecności SRP w jeziorach na terenie Pomorza, przez okres dwóch lat pobierano próbki wody z różnych głębokości. Identyfikacja oraz kompleksowa charakterystyka genomowa i fenotypowa wyizolowanych bakterii pozwoliła na wykrycie w badanych jeziorach obecności bakterii z rodziny *Pectobacteriaceae*. Co ciekawe, wśród wyizolowanych bakterii zidentyfikowano szczep należący do niedawno ustanowionego gatunku: *Pectobacterium aquaticum*. Szczep *P. aquaticum* IFB5637 wykazywał aktywność enzymów rozkładających składniki roślinnych ścian komórkowych (ang. *plant cell wall-degrading enzymes*, PCWDEs), w tym pektynaz, celulaz i proteaz. Wykazano także, iż w warunkach laboratoryjnych szczep *P. aquaticum* IFB5637 jest zdolny do maceracji tkanki bulw ziemniaka oraz liści cykorii. Uzyskane wyniki wskazują, że stosowanie wód śródlądowych do nawadniania pól, na których uprawiany jest ziemniak, może przyczyniać się do rozprzestrzeniania się bakterii pektynolitycznych i odnotowywania objawów chorobowych czarnej nóżki i mokrej zgnilizny na roślinach.

Kolejna część przeprowadzonych badań poświęcona była identyfikacji determinant wirulencji u *P. aquaticum* i *Dickeya solani*. Jednym z czynników warunkujących wirulencję bakteryjnych patogenów roślin są lipopolisacharydy zewnątrzkomórkowe (ang. *lipopolysaccharide*, LPS), które odgrywają ważną rolę na wczesnym etapie interakcji roślina-patogen. W kolejnych badaniach skupiono się na identyfikacji struktury i składu cukrowej części LPSu (ang. *O-polysaccharide*, OPS) *P. aquaticum* IFB5637. W wyniku przeprowadzonych analiz

ustalono, że w skład OPS wchodzą pentamery zbudowane z dwóch powszechnie znanych heksoz: mannozy i glukozy, oraz unikatowy cukier abekoza, który w tej pracy został po raz pierwszy zidentyfikowany w komórkach bakterii z rodziny *Pectobacteriaceae*.

Zdolność bakterii z gatunku *D. solani* do wywoływaniu objawów chorobowych wynika z efektywnej produkcji enzymów degradujących ścianę komórkową roślin, nazywanych PCWDEs. Pomimo dużej jednorodności genetycznej szczepów *D. solani*, niektóre z nich wykazywały skrajnie różną zjadliwość. Dwa szczepy *D. solani* różniące się zdolnością do wywoływania objawów chorobowych na roślinach wykorzystano w badaniach nad identyfikacją czynników wirulencji. Przeprowadzone badania koncentrowały się na analizie metylomów i transkryptomów dwóch szczepów *D. solani*: wirulentnego IFB0099 i niewirulentnego IFB0223. Bioinformatyczna analiza metylacji DNA nie wykazała istotnych różnic między profilami metylacji DNA badanych szczepów. Natomiast analiza profili transkrypcyjnych metodą RNA-Seq wykazała różnice w poziomie ekspresji szeregu genów pomiędzy porównywanymi szczepami. Niektóre z genów kodujących składniki systemu sekrecyjnego typu II (ang. *type II secretion system*, T2SS) (*gspJ, nipE*) oraz trzy z genów kodujących endo-pektynazy (*pelD, pelE, pelL*) wykazywały wyższy poziom stymulacji ekspresji w warunkach indukcyjnych w przypadku szczepu IFB0099 w porównaniu do IFB0223, co może przyczynić się do jego zwiększonej wirulencji. Dodatkowo, zwiększona ekspresja genów kodujących białka budujące wici w IFB0099 (*flgC, flgB, flgD, fliG, flgG, flgF, flhA, fliA*) może mieć wpływ na zwiększoną mobilność tego szczepu.

Stopień nasilenia chorób wywołanych przez bakteryjne patogeny roślin jest zależny od odporności rośliny gospodarza, wirulencji patogena oraz warunków środowiskowych. Dlatego zbadano, czy rozwój objawów chorobowych wywołanych przez bakterie z rodzajów *Dickeya* i *Pectobacterium* może być uzależniony od właściwości gleby, a zwłaszcza składu jej mikrobioty bakteryjnej. W literaturze pojawia się termin „gleba supresyjna", który jest używany do opisania gleby, w której na wzrastających roślinach nie występują objawy choroby lub są minimalne, nawet w obecności patogena. W przeciwieństwie do „gleby supresyjnej", jako „glebę niesupresyjną" definiuje się glebę, której skład mikrobiologiczny sprzyja rozwojowi objawów chorobowych na roślinach. Na podstawie wcześniejszego monitoringu częstości występowania objawów czarnej nóżki i mokrej zgnilizny na plantacjach ziemniaka, wytypowano dwa pola z glebą o właściwościach supresyjnych i niesupresyjnych. Przeprowadzone analizy wykazały, że gleba supresyjna i niesupresyjna charakteryzowała się podobnymi właściwościami fizykochemicznymi. Natomiast sekwencjonowanie amplikonów dla genów kodujących 16S rRNA wykazało różnice w składzie mikrobioty bakteryjnej między dwoma analizowanymi rodzajami gleby. Wykazano, że przedstawiciele rodzajów *Bacillus, Acidobacterium* i *Gaiella* są znacznie liczniejsi w badanej glebie supresyjnej w porównaniu do gleby niesupresyjnej.

Podsumowując, przeprowadzone badania wzbogacają naszą wiedzę na temat złożonych interakcji między ekonomicznie istotnymi patogenami roślin z rodzajów *Dickeya* i *Pectobacterium* a środowiskiem i roślinami gospodarczymi. Wykonana analiza otwiera nowe

perspektywy dla efektywniejszego kontrolowania rozprzestrzeniania się chorób roślin, powodowanych przez bakterie pektynolityczne.

3. A summary of the obtained results:

Introduction

Plant pathogenic bacteria, and among them soft rot *Pectobacteriaceae* (SRP), are responsible for a substantial damage associated with the soft rot of many economically important crops, vegetables and ornamental plants worldwide in addition to blackleg on potato (Gijsegem et al., 2021a; Toth et al., 2011). All strains currently designated as SRP were initially classified to the *Bacillus carotovorus* species (Jones, 1901). Subsequently, these pathogens were reclassified to the genus *Erwinia* and two separate species were distinguished in the family *Enterobacteriaceae*: *Erwinia carotovora* and *Erwinia chrysanthemi* (Burkholder et al., 1953). In 1998, the members of the above-listed species were reassigned to the newly created genus *Pectobacterium* as either *Pectobacterium carotovorum* or *Pectobacterium chrysanthemi*, respectively (Hauben et al., 1998). The current genus *Dickeya* was established in 2005 and all the strains designated as *Pectobacterium chrysanthemi* have been transferred to it (Samson et al., 2005). Due to a continuous progress in genetic-based classification methods, 13 *Dickeya* and 22 *Pectobacterium* species are delineated nowadays (Toth et al., 2021a; Hong et al., 2023; Boluk et al., 2022).

SRP belong to gram-negative microorganisms, facultatively anaerobic, non-spore-forming and motile (Perombelon and Kelman, 1980). The typical cell size for *P. carotovorum* is approximately 0.5-0.7 μm in width and 1.2-2.2 μm in length (Ni et al., 2010). The cells of *Dickeya* spp. on the other hand have been estimated to range from 1.5 to 3.6 μm in width and 0.6-1.1 μm in length (Rungnapha et al., 2008). Regarding the biochemical profiles, SRP exhibit catalase activity, glucose fermentation, nitrate reduction, and the production of β-galactosidase and H_2S. Moreover, they can utilize various sugars including L-arabinose, D-galactose, D-glucose, glycerol, D-mannose, D-ribose, and sucrose. However, they do not generate urease or produce acid when utilizing adonitol (De Boer and Kelman, 2001). Moving to the differences, the distinguishing traits of the *P. atrosepticum* strains include their ability to use α-methyl-D-glucoside, generate reducing sugars from sucrose, grow in a medium containing 5% NaCl, and their inability to grow at 37°C (De Boer and Kelman, 2001; Gardan et al., 2003). On the other hand, *Dickeya* spp. exhibit phosphatase activity, the capacity to synthesize indole, and susceptibility to erythromycin. They have the capability to grow at various temperatures, spanning from 21°C to 41°C, with an optimal growth temperature of approximately 32°C (Du Raan et al., 2016). In the past, the metabolic properties or biochemical profiles were the basis for taxonomic classification of bacterial phytopathogens, but currently these features are mainly used to assess biodiversity among the strains already assigned to specific taxa.

The diseases caused by SRP include blackleg and soft rot. The symptoms of blackleg involve blackening of the stem base, stunting, wilting, chlorosis of the leaves, necrosis, decline in yield, and even decay of the whole potato plant. On the other hand, soft rot is manifested by soft, wet, rotten, tan or cream-colored tissues. The affected tissue parts are sharply delineated from the healthy ones by dark brown or black margins (Perombelon and Kelman, 1980).

Dickeya and *Pectobacterium* spp. cause significant economic losses in crucial crops across the globe. Due to this fact, these pests have been listed among the 10 most destructive plant pathogenic bacteria in 2010 (Mansfield et al., 2012). Specifically, the presence of SRP in farmlands leads to crop losses reaching up to $420 million annually (Birch et al., 2012), with the highest economic impact on the potato production sector (Toth et al., 2011). For instance yield reductions in Israel amounting from 20% to 25% as observed on different potato varieties, have been attributed to the occurrence of *Dickeya* spp. (Tsror et al., 2009). Interestingly, other work reported the following contributions to the overall SRP-triggered damage as analysed in terms of diverse potato production sectors: the seed potato sector accounts for 32% of the losses, the table potato sector 43%, and the processing potato sector 25%. If studied with regard to the impact per hectare, the seed potato sector suffers from the greatest damage, with losses being 2.5 times higher than these recorded in the table potato sector, while the processing potato sector is affected in a manner 2.9 times lower than the table potato sector (Dupuis et al., 2021).

From an epidemiological perspective, it is extremely important that despite significant economic losses and the widespread presence of pectinolytic bacteria, there are currently no effective plant protection methods against these pathogens (Czajkowski et al., 2011). Until now, examination of potato seeds for latent infections with *Dickeya* and *Pectobacterium* spp. has been considered the most efficient approach for protecting crops (Toth et al., 2011; van der Wolf et al., 2021). Various methods, either chemical or biological, were also tested. Examples of compounds of plant protective properties include organics such as hydroxyquinoline and 5-nitro-8-hydroxyquinoline (Mills et al., 2006). In turn, biological control against pectinolytic bacteria could rely on specific bacteriophages (Czajkowski et al., 2016). Biocontrol strategies involve utilizing antagonistic organisms to inhibit pathogen populations through antibiosis, nutrient depletion, or triggering the plant systemic resistance. To exemplify, fluorescent *Pseudomonas* spp. if applied to potato tubers successfully decrease the populations of blackleg and soft rot bacteria present on the roots and inside subsequent generations of tubers (Kloepper, 1982). Another example is *Bacillus subtilis* BS 107, a biocontrol agent selected to combat the bacteria responsible for soft rot and blackleg (Sharga and Lyon, 1998). Moreover, it appears that physical control methods may compete with biological and chemical approaches. The physical measures utilized to manage tuber soft rot infection rely on the use of hot water or steam (Robinson and Foster, 1987; Shirsat et al., 1991), dry hot air (Bartz and Kelman, 1985), as well as UV and solar radiation (Bdliya and Haruna, 2007). It should be emphasized, however, that the methods used to protect plants against SRP are still very few, and usually their effectiveness is too low to ensure successful prevention of the spread of disease-causing microorganisms in the environment. Until now, the examination of potato seeds for latent infections caused by *Dickeya* spp. and *Pectobacterium* spp. has been considered the most effective plant protection strategy (van der Wolf et al., 2021).

It is worth to stress that the infected seed potatoes are the most important source of disease, however *Pectobacterium* spp. and *Dickeya* spp. can also reach a susceptible host plant from

various environmental sources, *e.g.* soil or water (Pérombelon and Salmond, 1995). Bacteria can be transmitted to the healthy plants from soil through wounded tissue or natural openings (Toth et al., 2021b). The survival of *Pectobacterium* and *Dickeya* spp. in the soil is influenced by a wide range of biotic and abiotic factors. The literature data indicate that the increased soil moisture significantly prolongs the viability of SRP (Burr and Schroth, 1977; Powelson and Apple, 1984). Moreover, bacteria from the genus *Pectobacterium* spp. seem to survive longer in soil than *Dickeya* spp. (Toth et al., 2021b). According to the research conducted in the Netherlands, when SRP were introduced to various soils at a temperature of 6°C and 50% moisture, *Dickeya* isolates obtained from potato and hyacinth demonstrated a survival period of no more than 7 days. In comparison, the survival period recorded for *Pectobacterium* spp. isolates under similar conditions amounted up to 42 days (van der Wolf et al., 2009).

Moving to the next source of SRP, these microorganisms have been detected in various waterways worldwide, including Australia, Finland, France, Malaysia, Poland, the United Kingdom, Spain, Switzerland, and the United States (Cahill et al., 2010; Hugouvieux Cotte Pattat et al., 2019; McCarter-Zorner et al., 1984; Harrison et al., 1987; Laurila et al., 2008; Laurila et al., 2010; Oulghazi et al., 2019a, 2019b; Palacio-Bielsa et al., 2010; Parkinson et al., 2014a; Pédron et al., 2019; Potrykus et al., 2016; Sueno et al., 2014). For example, from 572 surface water samples tested in southern Scotland and Colorado, 439 showed the presence of pectinolytic bacteria. Interestingly 95% of the obtained isolates were identified as *Pectobacterium* spp. (McCarter-Zorner et al. 1984). On the other hand, surveys of rivers in Finland disclosed *Dickeya* spp. as the dominating SRP genus in this environmental niche (Laurila et al., 2008). Moreover, the frequency of detection of *Pectobacterium* spp. and *Dickeya* spp. tended to increase in rivers located close to the arable land, suggesting that irrigation water can serve as a potential source of inoculum (Gijsegem et al., 2021a). Finally, a two-year investigation of the presence of SRP in the Durance river in southeastern France revealed a higher frequency of the isolations of *Pectobacterium* spp. compared to *Dickeya* spp. strains (Moussa et al., 2022).

By now, in addition to soil and water, many other SRP sources have been described. These bacteria were isolated so far from weeds, plant debris, agricultural machines, insects and nematodes (Pérombelon and Kelman 1980). SRP are characterized by a wide spectrum of plants they can infect, including the members of 16 families of the dicotyledonous species and 11 families of monocotyledonous plants (Ma et al., 2007). For example in the Netherlands, *Dickeya* spp. were identified on hyacinths (*Hyacinthus*) and daffodils (*Narcissus*), while the presence of *P. carotovorum* was noted on *Zantedeschia* sp. The affected plants exhibited typical symptoms of wilting, soft rot, and decline (van Doorn et al., 2011). As mentioned above, the use of contaminated agricultural machinery poses a significant threat associated with the spread of *Dickeya* spp. and *Pectobacterium* spp. It was demonstrated that assuring cleaning and proper sanitization of the equipment utilized in planting, spraying, and grading significantly lowered the likelihood of introducing soft rot bacteria to the uninfected seed lots (Pérombelon 2002;

Pérombelon and Kelman 1980). In the case of insects, data on their involvement in spreading *Pectobacterium* spp. and *Dickeya* spp. date back to 1920 (Toth et al., 2021b). For example, one of the studies revealed that SRP were commonly found in insects of *Diptera* order, captured in diverse potato fields across six latitudes and varying climate zones in Norway (Rossmann et al., 2018). Apart from insects, the presence of SRP has been associated with various nematodes. In more detail, *P. atrosepticum* can endure the digestion process in nematodes and be released into the surrounding environment as was demonstrated on the *Caenorhabditis elegans* model (Nykyri et al., 2014).

The pathogenicity-related potential of SRP is linked with efficient regulation, production and secretion of numerous virulence factors, predominantly plant cell wall degrading enzymes (PCWDEs), but also toxins and plant hormones (Davisson et al., 2013; Jiang et al., 2016; Toth et al., 2006; Charkowski et al., 2012). To efficiently decompose the plant cell wall and release valuable nutrients *Pectobacterium* spp. and *Dickeya* spp. secrete pectinases, proteases and cellulases (Boccara et al., 1994) among others enzymes. Pectinases, including endopectate lyases, rhamnogalacturonate lyases in addition to pectin lyases and methylesterase (Gijsegem et al., 2021b), are major contributors to the observed maceration symptoms.

Besides an arsenal of enzymes, effective secretion systems are needed to transfer these proteins outside the bacterial cell (Glasner et al., 2011; Pedron et al., 2014). In SRP, T1SS handles the secretion of extracellular proteases, while the T2SS (Type 2 Secretion System) is responsible for the transmembrane shift of the majority of extracellular pectinases and cellulases (Charkowski et al., 2012). In addition, *Dickeya* spp. and *Pectobacterium* spp. possess several other secretion systems: T3SS, T4SS, T5SS and T6SS. SRP exhibit a common set of T3SS effector molecules, including the HrpN and HrpW proteins with harpin-like properties, as well as a member of the AvrE/DspE family (Gijsegem et al., 2021b). Greater variability was noted in the effectors of T4SS, T5SS, and T6SS. SRP seem to possess two varieties of T4SS, linked either to plasmid conjugation and DNA interactions or connected to protein secretion (Wallden et al., 2010). T5SS and T6SS, on the other hand, both play roles in contact-dependent competition systems, functioning within and between the representative of individual species (Gijsegem et al., 2021b).

Efficient colonization of the plant host requires also additional virulence factors, like flagella, lipopolysaccharides or exopolysaccharides, enabling movement or bacterial adhesion, as well as intermediary factors essential for adaptation to stressful conditions within the plant host tissue, allowing for the overcoming of oxidative stress, iron deficiency or the presence of toxic compounds (Reverchon et al., 2016).

In summary, *Dickeya* spp. and *Pectobacterium* spp. belong to the economically most important bacterial plant pathogens significantly affecting global potato production. Nevertheless, there are no control methods to efficiently tackle with blackleg and soft rot, as only preventative measures are available. This study introduces new insights into understanding the virulence and spread of *Dickeya* spp. and *Pectobacterium* spp. Moreover, among the herein set aims there was

elucidation of the structure of bacterial microbiota responsible for soil suppressiveness against SRP. The presented research may be the first step for the preparation of complex, effective biological formulations that may be applied as biocontrol agents for agricultural purposes.

Aims

The following aims have been set within the herein presented PhD thesis:

- **Assessment whether Polish waterways may be the source of bacteria from the family *Pectobacteriaceae*, which can cause symptoms of soft rot and blackleg diseases on potato.**
- **The search for virulence determinants in *Pectobacterium aquaticum* and *Dickeya solani*.**
- **Application of metagenomics for elucidation of the differences in the structure of bacterial microbiota in suppressive and non-suppressive soils in relation to Soft Rot *Pectobacteriaceae.***

Results

The results of the herein presented PhD thesis addressing the first research objective, *i.e.* 'Assessment whether Polish waterways may be the source of bacteria from the family *Pectobacteriaceae*, which can cause symptoms of soft rot and blackleg diseases on potato', were published in an indexed scientific journal of an international reach:

Publication no 1: **Weronika Babińska**, Agata Motyka-Pomagruk, Wojciech Śledź, Agnieszka Kowalczyk, Zbigniew Kaczyński, Ewa Łojkowska. (2021). The first Polish isolate of a novel species *Pectobacterium aquaticum* originates from a Pomeranian lake. Int. J. Environ. Res. Public Health, 18, 5041. doi: 10.3390/ijerph18095041. **IF_{2021} 4.614, $MNiSW/MEiN_{2021}$ 140**

The research group of Prof. Ewa Łojkowska has been monitoring the occurrence of *Pectobacterium* spp. and *Dickeya* spp. on potato fields in Poland since 1996. As a continuation of this research, it was decided to examine Pomeranian lakes to state whether these water reservoirs can be a source of *Pectobacterium* or *Dickeya* spp. and contribute to the spread of SRP in the environment. Nine lakes were examined during the two-years monitoring study and the water samples were collected by me as a qualified scuba diver from the depths of 0 m, 5 m, 10 m, 15 m and 20 m. In addition, the samples of bottom sediment from a depth of 20 m and the samples of water collected near the shore were also included in this analysis. It needs to be stressed that, to the best of my knowledge, this is the first study focused on examination of the occurrence of SRP at different depths in the surface water reservoirs.

The water samples, were inoculated on a semi-selective CVP medium (Crystal Violet Pectate Medium) and incubated at 28°C for 48h. Pits-forming isolates were cultured on TSA (Tryptic Soy Agar) until axenic cultures were obtained. Species identification was performed firstly with the Multiplex PCR (Potrykus et al., 2014b), which allowed for the identification of the *Dickeya* spp., *P. atrospeticum* and *P. carotovorum* group. Later on, to evaluate whether strains classified to the *P. carotovorum* group should be reassigned to *P. brasiliense*, a species-specific PCR (Duarte et al., 2004) was used. The IFB5637 pectinolytic isolate that originated from Jeleń lake, gave amplicons with PccF and PccR starters (Potrykus et al., 2014b) and in the species-specific PCR designed by Duarte et al. (2004). On the template of the DNA isolated from the strain IFB5637, fragments of highly-conserved housekeeping genes *dnaX* (Sławiak et al., 2009) and *recA* (Waleron et al., 2002) were amplified and sent for the Sanger sequencing service. After aligning the fragments of 535 bp of *dnaX* and 730 bp of *recA* genes, two independent Neighbour-Joining phylogenetic trees have been computed.

Based on the conducted phylogenetic analysis, the IFB5637 isolate showed the highest similarity to the *Pectobacterium aquaticum* strains available in the Genbank database (including type strain *P. aquaticum* CFBP 8637^{TS}) (Figure 1, Babińska et al., 2021, IJERPH, Publication no. 1). Regarding the single nucleotide polymorphisms (SNPs) detected in the *dnaX* gene fragments of

the *P. aquaticum* isolates included in the analysis (CFBP 8637^{TS}, IFB5637, CFBP 8632, CFBP 8636, CFBP 8633, CFBP 8636, CFBP 8634), two SNP regions have been distinguished (at position 222 bp and position 451 bp) in which the C-T transition occurred in the herein identified *P. aquaticum* IFB5637. The sequence of the *recA* gene of IFB5637 was identical to that of the strain CFBP 8637^{TS}, CFBP 8636 and the other strains of *P. aquaticum* (CFBP 8632, CFBP 8633, CFBP 8636, CFBP 8634). **As a result of this study, an isolate from *Pectobacterium aquaticum* species, a taxon not detected before in Poland, was acquired from a Pomeranian lake**.

In order to examine biodiversity among the strains of *P. aquaticum*, genomic profiling was performed using ERIC primers (Varsalovic et al., 1994). The analysis showed a high similarity between the analysed *P. aquaticum* IFB5637 isolate and the reference *P. aquaticum* strains from renowned international bacterial collections: CFBP 8637^{TS}, CFBP 8632, CFBP 8633, CFBP 8634, CFBP 8635 and CFBP 8636 (Figure 2, Babińska et al., 2021, IJERPH, Publication no. 1).

Subsequently, the aim was to determine whether the *P. aquaticum* strains of water origin exhibit pathogenic potential and may pose a threat to potato production. The capacity of *P. aquaticum* IFB5637 to macerate plant tissue and the activities of the main virulence-associated features of this isolate were examined in comparison to the other representatives of SRP *i.e. P. brasiliense* (Pbr CFBP 6617, Pbr HAFL05), *P. carotovorum* (Pc SCRI 136, Pc IFB5369), *P. parmentieri* (Ppa SCC3193), and *P. atrosepticum* (Pba SCRI 1086). In more detail, the bacterial ability to macerate potato and chicory tissues besides the activities of crucial virulence determinants of soft-rotting pectinolytic bacteria such as pectinases, cellulases, proteases and lipases were revealed in this study. Finally, the bacterial abilities to produce siderophores and biofilm, as well as to swim and swarm were determined. The collected results showed that *P. aquaticum* IFB5637 was able to macerate potato tubers and chicory leaves, but exhibited lower virulence in comparison to the two tested *P. brasiliense* (CFBP 6617 and HAFL05) and two *P. carotovorum* (SCRI 136 and IFB5637) strains (Figure 3, Babińska et al., 2021, IJERPH, Publication no. 1). The diminished pectinase activity of *P. aquaticum* IFB5637 strain was linked with the limited ability of IFB5637 to decay potato and chicory tissues. Unexpectedly, the cellulase and protease activities of this strain were higher than those of *P. atrosepticum* strain isolated from the rotten potato tubers. **It is important to emphasize that the *P. aquaticum* IFB5637 turned out to be able to produce a wide spectrum of plant cell wall-degrading enzymes, including pectinases, proteases, cellulases and to cause plant tissue maceration** (Table 2, Babińska et al., 2021, IJERPH, Publication no. 1). *P. aquaticum* IFB5637 was also revealed to be able of moving by swimming or swarming, though this isolate showed lower biofilm formation capacity than the other tested *Pectobacteriaceae* strains. Moreover, it showed higher, compared to the other SRP strains included in this study, iron chelating potency. Finally, the sugar composition of the O-polysaccharide (OPS), involving mannose, glucose and abequose constituents, of the bacterial LPS of *P. aquaticum* IFB5637 strain was determined. Importantly

abequose is a monosaccharide that was identified for the first time among the pectinolytic members of the *Pectobacteriaceae* family.

To the best of our knowledge, the herein described IFB5637 strain is the first representative of a recently established *P. aquaticum* species in Poland and originated from the waterways. It is also important to underline that in this research the water reservoirs were monitored for the presence of SRP at different depths for the first time. It was shown here that pectinolytic bacteria had been be isolated only from 0 m depth nearby the shore.

On this basis, it can be assumed that the use of irrigation water collected from higher depths instead of the surface, might contribute to limitation in the spread of pectinolytic bacteria on farmlands.

The results of the herein presented PhD thesis addressing the second research objective, *i.e.* 'The search for virulence determinants in *Pectobacterium aquaticum* and *Dickeya solani*', were either published (Publication no. 2) or submitted (Manuscript no. 1) to indexed scientific journals of an international reach:

Publication No. 2: Agnieszka Kowalczyk, Nicola Szpakowska, **Weronika Babińska**, Agata Motyka-Pomagruk, Wojciech Śledź, Ewa Łojkowska, Zbigniew Kaczyński (2022). The structure of an abequose - containing O-polysaccharide isolated from *Pectobacterium aquaticum* IFB5637. Carbohydr Res. 522, 108696. doi: 10.1016/j.carres.2022.108696. **IF_{2022} 3.34, $MNiSW/MEiN_{2022}$ 100**

The identification of an abequose, a unique monosaccharide in the *Pectobacteriaceae* family (Babińska et al., 2021, IJERPH, Publication no. 1), prompted further studies on the variable part of LPS in this group of phytopathogenic bacteria. Therefore, in Publication no. 2 elucidation of the exact chemical structure of the OPS of *P. aquaticum* IFB5637 was accomplished. Additionally, a structural comparison was conducted between the OPS of *P. aquaticum* IFB5637 and the previously identified OPS of other representatives of the *Pectobacterium* genus. The attention was focused on the structure of LPS, which consists of lipid A, the inner and outer core, and a most variable region OPS, due to the fact that this molecule plays a role of an accessory virulence factor in SRP. In particular LPS is engaged in the first stage of SRP infection, which involves the recognition of the pathogen by the plant host. The importance of LPS for the pathogenicity of SRP has already been indicated by the research of Toth et al. (1999), in which it was proven that a mutation in the gene important for the biosynthesis of lipopolysaccharides reduces the virulence of the bacteria from the closely related species *Pectobacetrium atrosepticum* (formerly *Erwinia carotovora* subsp. *atroseptica*).

Initially, in order to determine the chemical structure of the OPS of *P. aquaticum* IFB5637, there was a need to obtain a sufficient amount of bacterial biomass. For this purpose, *P. aquaticum* IFB5637 was recovered from a frozen stock and plated on a TSA medium in a reductive manner. Post 24h incubation at 28°C, a single colony was picked to inoculate 200 ml of the TSB medium. After 24h incubation at 28°C the so-prepared precultures were used for inoculation of 2000 ml of the TSB medium. The latter cultures were conducted for 48h at 28°C with 130 rpm shaking. Then, the cells of *P. aquaticum* IFB5637 were harvested by centrifugation (6,915.34×g for 10 min). The above-described methodology was repeated until a sufficient amount (minimum 80 g) of bacterial biomass was reached. The cells of *P. aquaticum* IFB5637 were kept at -20°C for further analyses.

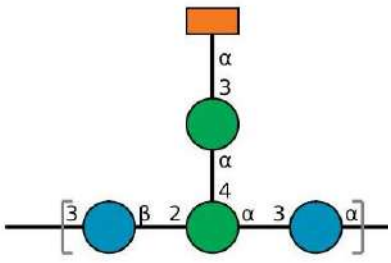
Subsequently, the LPS was isolated from the cells of *P. aquaticum* IFB5637. For this purpose two extraction methods were implemented. Initially, the PCP (Phenol-Chloroform-Petroleum ether) technique was used (Galanos et al., 1969), which relies on subjecting bacterial biomass to the mixture of phenol, chloroform, and petroleum ether mixed in a 2:5:8 ratio (v/v/v). Due to the

fact that no precipitate formed during the above-described extraction, it was assumed that the tested bacterial strain does not produce a rough type of LPS. Therefore, another extraction technique dedicated for isolation of a smooth LPS that is based on mixing dry bacterial cells with hot phenol and water was used (Westphal and Jann, 1965). The water phase obtained from the extraction was dialyzed, lyophilized and finally subjected to enzymatic digestion to remove proteins and nucleic acids.

In order to prepare the OPS, the LPS extract underwent hydrolysis using 1% acetic acid to cleave lipid A from the sugar component. A portion of the resultant fraction was dissolved in deionized water and subjected to gel-permeation chromatography (GPC). Afterwards, the fractions obtained from the GPC were freeze-dried, and the proton nuclear magnetic resonance spectra (1H NMR) were recorded. Using this data, a fraction of high-molecular OPS mass was identified and chosen for subsequent structural analyses.

Finally, the chemical structures and the number of different sugar residues included of the isolated OPS were described in collaboration with the research group of Professor Zbigniew Kaczyński. The results of sugar analysis revealed the presence of two hexoses in a 1:1 molar ratio, which were identified as mannose and glucose. In order to assign the substitution positions of the identified monosaccharides, a methylation analysis was performed, which showed a presence of three partially methylated derivatives of alditol acetate: two different 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol and 1,2, 4,5-tetra-*O*-acetyl-3,6-di-*O*-methylhexitol in an approximate molar ratio of 2:1:1.

The interpretation of the recorded NMR spectra led to a conclusion that the O-antigen of the analysed strain consists of five sugar units (Figure 1, Kowalczyk et al., 2022, Carbohydr Res, Publication no. 2). Besides the earlier identified mannoses and glucoses, a 3,6-dideoxy-d-xyl-hexose residue (abequose) was identified (Table 1, Kowalczyk et al., 2022, Carbohydr Res, Publication no. 2). Notably, this molecule has not been identified in the conducted sugar analysis as it broke down under classical analysis conditions. **The OPS obtained from *P. aquaticum* IFB5637 was revealed in this work by applying chemical analyses and NMR spectroscopy to be composed of the repeating pentasaccharide units:**



abequose glucose mannose

(Kowalczyk et al., 2022, Carbohydr Res, Publication no. 2)

So far, the OPS structures of more than 10 strains from the *Dickeya* spp. and *Pectobacterium* spp., including another isolate from water (*P. versatile* CFBP6051^T), have been characterized by

our research team in collaboration with group of Professor Zbigniew Kaczyński (Czerwicka et al., 2011; Kowalczyk et al., 2023; Ossowska et al., 2016; Ossowska et al., 2022; Szulta et al., 2023). However, the abequose residue was identified as a unique component of the OPS in the *Pectobacteriaceae* family for the first time. **It is worth mentioning that the abequose has not been identified in the OPS of any other plant pathogenic bacteria so far.**

The above-mentioned results in addition to the literature data (Lukianova et al., 2020; Senchenkova et al., 2003; Senchenkova et al., 2005; Shneider et al., 2020) indicated high structural variations in the OPS of the members of the *Pectobacteriaceae* family. Further studies, including construction of the mutants, incapable of producing LPS are required to elucidate the relationship between the LPS structure and the virulence of bacteria from the genera *Dickeya* and *Pectobacterium*.

Manuscript No. 1: **Weronika Babińska-Wensierska**, Agata Motyka-Pomagruk, Alessio Mengoni, George C. diCenzo, Ewa Łojkowska (2023). The search for the foundations of virulence in an economically significant plant pathogen *Dickeya solani via* transcriptomic profiling. Submitted for publication

Several European and American research groups have been working to elucidate which virulence factors contribute to the pathogenicity of bacteria from the *Pectobacteriaceae* family. Our team is concentrated on the virulence factors in the *Dickeya solani* species (Golanowska et al., 2018; Motyka-Pomagruk et al., 2020; Potrykus et al., 2014a, 2018, 2020). Bacteria from the *D. solani* species efficiently spread throughout Europe and cause higher losses in potato production than bacteria from the other *Dickeya* species (Toth et al., 2011). The former studies of the group of Professor Ewa Łojkowska revealed notable differences in the virulence of *D. solani* strains of diverse origin and year of isolation (Golanowska et al., 2017, 2018; Motyka-Pomagruk et al., 2020; Potrykus et al., 2014a). Most of all, the *D. solani* IFB0099 and IFB0223 strains stood out, as they showed diverse abilities to macerate potato tissue in addition to pectinase and protease activities. However, the comparative genomics studies conducted so far, along with pangenome analysis, have not yet provided clear answers to explain the differences in the virulence-related phenotypes between *D. solani* IFB0099 and IFB0223 strains (Golanowska et al., 2018; Motyka-Pomagruk et al., 2020). Therefore, searching for delineation of the differences in the pathogenic phenotypes of *D. solani* strains by the means of genomic DNA methylation analysis and transcriptomic profiling was the subject of the herein presented Manuscript no. 1. To reach the above-listed aim, the two mentioned earlier *D. solani* strains (highly virulent IFB0099 and low virulent IFB0223) differing in the plant maceration potencies towards potato and chicory and activities of PCWDEs (Potrykus et al., 2014a; Golanowska et al., 2018) were included in the presented research.

First of all, the DNA methylation analysis was performed to state whether diverse methylation patterns may be the reason for variation in the regulation of the expression of genes encoding virulence factors. To accomplish this objective, the genomes of *D. solani* strains IFB0099 and IFB0223 were sequenced on the PacBio platform. The sequencing service was outsourced to an external company, BaseClear, (Golanowska et al., 2018). By applying bioinformatic tools, *i.e.* ipdSummary version 2.4 and motifMaker, identification of the methylated sites and motifs in these two strains was achieved. In the genomes of both *D. solani* strains, in total three methylated motifs have been discovered and all of them turned out to be N6-methyladenosine (m6A) modifications (Table 1, Babińska-Wensierska et al., 2023, Manuscript no. 1). **On the basis of the obtained results it can be concluded that the DNA methylation patterns of *D. solani* IFB0099 and IFB0223 were very similar and cannot explain the significant differences in their virulence.**

To further investigate whether the differences in virulence are linked with diversified gene expression patterns, we conducted transcriptomic profiling of the *D. solani* IFB0099 and IFB0223 strains using RNA-Seq analysis. For this purpose, *D. solani* IFB0099 and IFB0223 were cultured in a minimal medium either supplemented or not with PGA. Supplementation of media with PGA, a structural element of the plant cell wall, mimics a more advanced stage of the infection process and is commonly applied for induction of expression of genes encoding pectate lyases under laboratory conditions (Hugouvieux-CottePattat et al., 1992; Łojkowska et al. 1995; Potrykus et al. 2014a). The bacterial cultures of *D. solani* IFB0099 and IFB0223 were grown in either regular M9 medium (non-induced conditions) or M9 medium with 4 g L^{-1} PGA (induced conditions) and incubated for 48h at 28°C with 130 rpm shaking.

Then, the total RNA was extracted by using the Bead-Beat Total RNA Mini kit (A&A Biotechnology, Łodz, Poland). The isolated RNA was then purified and concentrated using Clean-Up RNA Concentrator and Total RNA Mini Concentrator kits (A&A Biotechnology, Łodz, Poland). After isolation of the RNA and conducting the RNA-Seq on a MiSeq platform by an external sequencing company Genomed, the acquired raw sequencing data were trimmed using the Trimmomatic tool (Bolger et al., 2014). Afterwards, quality checks were performed using the FASTQC program (Andrews et al., 2010). Subsequently, the reads were mapped to the reference genome using Bowtie2 (Langmead et al., 2012). These mapped reads were used for quantitative analysis in the R programming environment (RStudio version 2021.09.0), which involved identification of differentially expressed genes (DEGs) between the *D. solani* strains IFB0099 and IFB0223 cultured under the two investigated conditions.

Finally, an analysis of differences in the gene expression profiles of *D. solani* IFB0099 and IFB0223 grown in induced and non-induced conditions was performed. The induction with PGA in both strains led to an increased expression of genes associated with plant cell wall and pectin degradation. These genes include endo-pectate lyases such as *pelD, pelE, pelI, pelB, pelL, pelC*, as well as rhamnogalacturonate lyases like *rhiE* (Tables 2-3, Babińska-Wensierska et al., 2023, Manuscript no. 1). Among the above-mentioned genes, *pelD, pelE, pelL* exhibited an elevated induction of expression due to the presence of PGA in the virulent strain (IFB0099) compared to the low virulent one (IFB0223) (Tables 2-3, Babińska-Wensierska et al., 2023, Manuscript no. 1). *D. solani* also produces enzymes known as pectin acetyl esterase (*peaY*) and pectin methyl esterases (*pemA*). In the case of IFB0099, their expression was stimulated to a higher manner with Log2F Fold Chain values of 1.57 and 1.92, compared to IFB0223 with Log2FC 0.84 and 1.79, respectively (Tables 2-3, Babińska-Wensierska et al., 2023, Manuscript no. 1). Moving on to proteases, which degrade proteins present in plant cell wall in addition to constituents of the plant defence systems (Gijsegem et al., 2021a), it was observed that the genes *prtG*, *prtB* and *prtC* encoding three proteases, demonstrate higher stimulation of expression in strain IFB0099 compared to strain IFB0223 (Tables 2-3, Babińska-Wensierska et al., 2023, Manuscript no. 1).

The other differences identified between the transcriptomes of IFB0099 and IFB0223 strains, which had been cultured under either induced or non-induced conditions, were associated with differential expression of genes encoding proteins important for bacterial mobility. In more detail, there were eight motility-associated genes (*flgC, flgB, flgD, fliG, flgG, flgF, flhA, fliA*) that showed higher induction of expression in the presence of PGA in the virulent strain (IFB0099) compared to the less virulent strain (IFB0223). Moreover, all these genes showed higher expression level in the induced compared to the non-induced conditions (Tables 2-3, Babińska-Wensierska et al., 2023, Manuscript no. 1).

Finally, I analysed differential expression of genes associated with the Type 2 Secretion System (T2SS). One of these genes *gspJ*, exhibited decreased expression rate under induced conditions compared to non-induced conditions. It is worth to notice that *gspJ* gene demonstrated significantly lower expression in strain IFB0223 compared to strain IFB0099 (with a log2 fold change of -0.54 for IFB0099 and -1.26 for IFB0223). Another gene encoding a significant effector of type 2 system the NipE protein, is involved in inducing extracellular necrosis (Kazemi-Pour et al., 2004). In both the IFB0099 and IFB0223 strains, the *nipE* gene displayed increased expression under induced conditions compared to the non-induced conditions (Tables 2-3, Babińska-Wensierska et al., 2023, Manuscript no. 1). Notably, the virulent strain (IFB0099) exhibited significantly higher induction of expression of *nipE* than the less virulent strain (IFB0223) (with a log2Fold Change of 1.46 for IFB0099 and 0.48 for IFB0223).

The herein presented study for the first time reports methylation and transcriptomic profiling among highly and low virulent *D. solani* strains. **No significant differences in the overall DNA methylation patterns were detected between the two *D. solani* strains with regard to their different level of virulence. On the other hand, discrepancies were observed in the gene expression patterns of IFB0099 and IFB0223 if they were grown under induced versus non-induced conditions, potentially leading to the identification of candidate genes that might be associated with the virulence of *D. solani*.** The high virulence of IFB0099 might result from an increased production and secretion of pectate lyses, properly functioning T2SS and increased mobility. Further molecular studies, including the construction and testing of mutants of IFB0099 in the pointed candidate genes, are necessary to confirm the impact of these genes on the virulence of this strain.

The results of the herein presented PhD thesis addressing the third research objective *i.e.* 'The application of metagenomics for elucidation of the differences in the structure of bacterial microbiota in suppressive and non-suppressive soils in relation to Soft Rot *Pectobacteriaceae*' were submitted to an indexed scientific journal of an international reach:

Manuscript No. 2: **Weronika Babińska-Wensierska**, Agata Motyka-Pomagruk, Marco Fondi, Agnieszka Emilia Misztak, Alessio Mengoni, Ewa Łojkowska (2023). Differences in the constituents of bacterial microbiota in relation to soil suppressiveness towards potato blackleg and soft rot diseases. Submitted for publication

The severity of plant diseases caused by pathogenic bacteria is determined by a complex interplay of factors. These include the resistance of the host plant, the virulence of the bacterial pathogen, and the prevailing environmental conditions. The speed of disease development in addition to the level of the resultant damage can vary significantly based on how these factors interact. For many years, the scientists have been suspecting that development of disease symptoms in crops is related to the so-called suppressiveness of the soil. In this regard, suppressive and non-suppressive soils are distinguished. The term suppressive soil is related to the specific composition of the soil microbiome and refers to a soil in which disease symptoms on the growing plants do not occur or are minimal, even in the presence of a virulent pathogen and a susceptible host plant. In contrast to the suppressive soil, scientists distinguish a non-suppressive soil, whose microbiological composition favours the development of certain disease symptoms on the growing plant. Therefore, in order to determine whether the progression of the *Dickeya* spp. and *Pectobacterium* spp.-driven infections may be associated with the composition of the bacterial microbiota of the soil collected from potato fields of diverse soft rot and blackleg incidence, a research described in Manuscript no. 2 was conducted.

Since 1996, the research group of Professor Ewa Łojkowska has been monitoring potato seed plantations for the presence of SRP. In certain locations, the growers consistently encountered no issues with blackleg and soft rot, while in the other cases, the problems associated with these diseases manifested annually. As in the fields located in Siemyśl (West Pomeranian Voivodeship) no blackleg symptoms were found during several years of monitoring, the soil from this field was selected as an example of suppressive soil. On the other hand, the non-suppressive soil originated from potato seed plantation in Bonin (West Pomeranian Voivodeship), in which blackleg was recorded repeatedly with the incidence of this disease in July equalling 2% and in August, being the month of harvest, increased to 20%. The selected soils were evaluated for physical and chemical properties and analysed for the presence of bacterial microbiota with a metagenomic approach. In addition, soil and potato tubers from the above-mentioned locations were collected during harvest for evaluation of the presence of pectinolytic bacteria.

Among the studied microelements and macroelements such as, N-NO_3, Cl, P, K, Ca, Na, Mg, B, Cu, Zn, Mn, Fe, organic carbon and organic substance, statistically significant differences between the investigated soils were observed only in the cases of Mg, Mn, organic C and the organic substance (Table 2, Babińska-Wensierska et al., 2023, Manuscript no. 2). Due to the small number of physicochemical differences observed between suppressive and non-suppressive soils in this analysis, the research focused on studying the composition of the bacterial soil microbiota.

Microbiological analysis of the suppressive and non-suppressive soils originating either from Siemyśl or Bonin, respectively, was performed. Soil and potato samples collected from the above-listed potato fields were tested for the presence of pectinolytic bacteria (*Dickeya* spp. and *Pectobacterium* spp.) by using the methodology of Potrykus et al. (2014b). Briefly, phosphate buffer was added to 1 g of soil or plant material and homogenized using a Bioreba hand homogeniser. Moreover, a pectate enrichment medium (PEM) was afterwards used to cultivate pectinolytic bacteria from the soil (48h at 28°C with shaking). Then, the selected dilutions of the resulting homogenate from soil or plant material or filtrate from soil were plated on CVP medium and incubated for 48h at 28°C. Bacterial colonies forming characteristic cavities on the CVP medium were further replated on CVP and TSA until an axenic culture state was reached. The cells of the isolated pectinolytic strains were lysed and subjected to a Multiplex PCR assay in order to achieve taxonomic identification. By this approach, it was possible to detect 4 *Pectobacterium* spp. isolates in the soil and 16 in the tissue of the mother tubers collected from the field with non-suppressive soil (Bonin). On the contrary, no pectinolytic bacteria were found in the field with suppressive soil (Siemyśl) (Table 3, Babińska-Wensierska et al., 2023, Manuscript no. 2).

Finally, sequencing of the 16S rDNA fragments amplified from the total DNA isolated from the suppressive and non-suppressive soil samples was performed. For this purpose, whole DNA isolation was performed using the commercially available NucleoSpin Soil Mini kit (Macherey-Nagel, Duren, Germany). The external company Genomed (Warsaw, Poland) prepared libraries and conducted the sequencing of the amplicons of the highly variable V3-V4 region of the 16S rDNA gene. Using Illumina v3 kit, the DNA was sequenced on the Illumina MiSeq instrument (Illumina, San Diego, USA). Bioinformatic analysis of raw data allowed for preliminary classification of the microbial sequences from soil using QIIME 2 (Bolyen et al., 2019). Subsequently, the reads were denoised using the DADA2 pipeline (Callahan et al., 2016), resulting in a collection of unique biological sequences known as Amplicon Sequence Variants (ASVs), which were classified as belonging to specific taxons by using a self-trained naive Bayes classifier (reference Silva 138 nr_v138 database) (Quast et al., 2013). At the end, the count tables for the annotated ASVs were then processed using the 'Phyloseq' R package (RStudio version 2021.09.0) (McMurdie et al., 2013).

The analysis revealed that the samples of suppressive and non-suppressive soil exhibited comparable microbial alpha diversity, but distinct bacterial community structures (beta diversity),

as evidenced by the principal coordinates analysis (PCoA) and PERMANOVA results (Figures 1, S2, Babińska-Wensierska et al., 2023, Manuscript no. 2). The most abundant bacterial phyla in both the tested soil types were Actinobacteriota, Proteobacteria, Acidobacteriota, Chloroflexi, Firmicutes, and Gemmatimonadota. At the class level, Actinobacteria, Alphaproteobacteria, Gammaproteobacteria, and Thermoleophilia were predominant in both soil types, with significant differences in the presence of Thermoleophilia between the suppressive and non-suppressive soil samples (Figures 2-3, Babińska-Wensierska et al., 2023, Manuscript no. 2).

Further analysis highlighted differential abundance between the tested soils in terms of the detected specific orders such as Acidobacteriales, Acetobacterales, Streptomycetales, and Bacillales (Figure 4, Babińska-Wensierska et al., 2023, Manuscript no. 2). The abundance of the above-mentioned orders was much higher in the case of the suppressive soil compared to the non-suppressive soil. Notably, *Bacillus* spp., *Rummeliibacillus* spp., *Acidobacterium* spp. and *Gaiella* spp. turned out to be notably more prevalent in the suppressive soil in contrast to the non-suppressive, suggesting their potential role in soil suppressiveness towards blackleg and soft rot diseases (Figure 5, Babińska-Wensierska et al., 2023, Manuscript no. 2).

This case study revealed differences in the occurrence of distinct bacterial taxa in soils, indicated either as suppressive or non-suppressive towards SRP. The abundance of the members of *Bacillus, Acidobacterium,* and *Gaiella* genera was significantly higher in the suppressive soil compared to the non-suppressive soil. Further advancement in this kind of research could enable the prediction of blackleg and soft rot incidences in potato fields by the determination of the structure of soil microbiota. Moreover, conducting such evaluations could aid in selecting the optimal fields for the cultivation of elite seed potatoes and these findings hint at the future development of effective biocontrol agents for protecting economically significant crops. This research is continued as a part of project in which I am the principal investigator entitled “Does the microbiome of arable soil influence development of blackleg and soft rot diseases caused by pectinolytic bacteria of the *Dickeya* and *Pectobacterium* genera?” (NCN PRELUDIUM 21, 2022/45/N/NZ9/01923, granted to Weronika Babińska-Wensierska).

Conclusions

1. **The first isolate of *Pectobacterium aquaticum* in Poland was acquired from Pomeranian lake.**

2. ***P. aquaticum* IFB5637, isolated from inland water is able to macerate potato and chicory tissues, therefore might pose a potential threat to potato cultivation.**

3. **The chemical structure of the repeating unit of the O-polysaccharide (two typical hexoses, including mannose and glucose, along with abequose) of the lipopolysaccharide isolated from *P. aquaticum* IFB5637 was established. The presence of an abequose in OPS has not yet been identified neither among the members of the *Pectobacteriaceae* family nor other plant pathogenic bacteria.**

4. **The analysis of the genome-wide DNA methylation pattern in *Dickeya solani* virulent IFB0099 and low virulent IFB0223 showed high similarity and cannot account for differences in the virulence of these strains.**

5. **Transcriptomic analysis indicated higher PGA-triggered stimulation of expression of genes encoding certain endo-pectate lyases, genes associated with the type 2 secretion system, and flagella-related genes in virulent *D. solani* IFB0099 compared to the low virulent IFB0223 strain, which may result in the increased virulence of IFB0099.**

6. **Analysis of 16S rDNA of bacterial microbiota isolated from suppressive and non-suppressive soil samples showed differences in abundance of bacteria from the genera *Bacillus, Acidobacterium* and *Gaiella*, which turned out to be identified more often in the suppressive soil than in the non-suppressive soil. The higher occurrence of bacteria from the above-mentioned genera may contribute to some extent to the suppressiveness of the tested soil.**

4. Attachments - Publications being the subject of the presented PhD thesis

Publication No. 1

The first Polish isolate of a novel species *Pectobacterium aquaticum* originates from a Pomeranian lake

Weronika Babińska, Agata Motyka-Pomagruk, Wojciech Śledź, Agnieszka Kowalczyk, Zbigniew Kaczyński, Ewa Łojkowska**.**

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Article

The First Polish Isolate of a Novel Species *Pectobacterium aquaticum* Originates from a Pomeranian Lake

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Abstract: Pectinolytic bacteria from the genus *Pectobacterium* cause high economic losses in various crops, vegetables, and ornamentals including potato. Thus far, these strains have been isolated from distinct environments such as rotten or asymptomatic plants, soil, and waterways. The prevalence of soft rot *Pectobacteriaceae* in different depths of Pomeranian lakes was performed by a qualified scuba diver over 2 years of monitoring. It allowed for the isolation and broad characterization of a strain from the newly established species *Pectobacterium aquaticum*. Phylogenetic analysis on the sequences of *dnaX* and *recA* genes revealed the highest similarity of this strain to *P. aquaticum* CFBP 8637^{T}. In addition to the determination of analytical profile index (API 20E), we discovered that this strain possesses a smooth form of a lipopolysaccharide with O-polysaccharide consisting of mannose, glucose, and abequose. Moreover, the characterized strain, described as *P. aquaticum* IFB5637, produced plant-cell–wall-degrading enzymes, such as pectinases, cellulases, proteases, and was capable of macerating potato and chicory tissues under laboratory conditions. In view of more frequent irrigation of seed potato fields resulting from the ongoing climate warming, it is important to monitor the occurrence of potential disease-causing agents in natural waterways.

Keywords: *Pectobacteriaceae*; pectinolytic bacteria; soft rot; blackleg; monitoring; natural waterways

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1. Introduction

Bacteria classified to the *Pectobacterium* genus are Gram-negative, non-sporulating, facultative anaerobes [1]. Members of this genus joined the newly established *Pectobacteriaceae* family [2] due to the fact of their genomic characteristics and their ability to produce pectinolytic enzymes. These bacteria cause disease symptoms of blackleg on potato plants and soft rot on potato tubers and other vegetables, e.g., tomatoes, peppers, chicory, and ornamental plants [3–5]. The virulence of these pathogens results from the production of large quantities of plant-cell-wall-degrading enzymes (PCWDEs), i.e., pectinases, cellulases, and proteases that are secreted outside bacterial cells and allow for efficient maceration of plant tissue [6]. Apart from the above-listed PCWDEs, pectinolytic bacteria possess an effective iron chelating system that enables iron accumulation and survival in spite of the limited availability of this element [7]. In addition, motility turned out to be highly relevant for the pathogenesis of *Pectobacterium* spp. [8,9], as by these means bacteria are able to enter the plant through wounds or natural openings, which leads to successful colonization and maceration of the host tissue.

It needs to be underlined that bacteria belonging to the genus *Pectobacterium* are highly heterogeneous [10–13] and differ in their ability to cause disease symptoms on diverse plant species [14] under varying temperature conditions [15]. For example, *Pectobacterium atrosepticum* is responsible for disease outbreaks only under temperate climate [1,12], while

Pectobacterium brasiliense is capable of triggering symptomatic infections in tropical and subtropical regions [16,17]. On the other hand, *Pectobacterium parmentieri* (until 2016 *Pectobacterium wasabiae*) was isolated from the affected plants in many European countries with various climate conditions (e.g., Norway, Poland, and Spain), in addition to such distant geographical areas such as New Zealand, South Africa, and Canada [10,11,13,18–20]. Referring to the broad host range of *Pectobacterium* spp., *Pectobacterium carotovorum* causes disease symptoms on potato, other vegetables, and ornamentals [21,22], while, for instance, *P. brasiliense* was observed to trigger soft rot in *Capsicum annum* L., *Ornithogalum* spp. and *Daucus carota* subsp. *sativus* [23].

The high significance of *Pectobacterium* spp. is underlined by enclosing these microbes in the top 10 list of bacterial plant pathogens based on scientific/economic importance [24]. Especially, notable annual economic damage results from the activity of *Pectobacteriaceae* in the potato production sector [1]. From an epidemiological point of view, it is extremely important that in spite of high economic losses and broad host range associated with pectinolytic bacteria, there is a lack of effective plant protection strategies against these phytopathogens [25].

Taking into account that *Pectobacterium* spp. spread by the latently infected seed tubers, they are classified as seed borne pathogens. In addition, soil, water, plant remains, insects, and nematodes, and agricultural machines are considered as important sources of pectinolytic bacteria [25–29]. Moreover, excessive irrigation and mechanical damage during harvest or insufficient ventilation in storage aggravate the severity of the resultant disease symptoms [4]. There were several reports suggesting that surface waters may be a potential sources of *Pectobacterium* spp. [30]. These communications considered the presence of *P. carotovorum* and *P. atrosepticum* in waters originating from drains, ditches, streams, rivers, and lakes in the USA and Scotland [31–33]. Moreover, *Pectobacterium versatile* was detected in waterways such as an alpine river stream, a river crossing arable land, and downstream from an irrigation canal [34]. Recently, a novel bacterial species, namely *Pectobacterium aquaticum* has been established [35]. By now, isolates from this taxonomic group have been isolated solely from three different water reservoirs in southeastern France (2015–2016) [35]. The abovementioned reports confirm that surface waters, which may potentially be used for irrigation purposes, are likely to harbor pectinolytic bacteria.

Our research group has been monitoring the occurrence of pectinolytic bacteria on seed potato plantations in Poland since 1996 [5]. The members of *Pectobacteriaceae* were also detected in some of the tested waterways in our country [36]. Recently, we undertook monitoring of the prevalence of pectinolytic bacteria at different depths in nine Pomeranian lakes. On this basis, we describe the first isolation, identification, and characterization of *P. aquaticum* strain originating from a natural water reservoir in Poland.

2. Materials and Methods

2.1. Water Samples Collection

Water samples were collected from the following 9 lakes located in the Pomeranian region in northern Poland: Wiejskie (54°01′14′ N 17°17′13′ E) (54°02′10.6″ N 17°28′02.9″ E), Czarne Dabrowno (54°07′44.1′ N 17°36′08.2′ E) (54°13′18.48″ N 17°60′58.18″ E), Wdzydze (53°58′31′ N 17°54′19′ E) (53°57′18.1″ N 17°54′19.9″ E), Biale (54°22′32′ N 18°11′13′ E) (51°49′80.7″ N 23°53′56.5″ E), Radunskie (54°16′11′ N 18°01′16′ E) (54°16′72.2″ N 18°16′66.8″ E), Klodno (54°19′02′ N 18°06′32′ E) (54°32′05.44″ N 18°10′99.80″ E), Jelen (54°12′04.4′ N 17°31′31.5′ E) (54°12′04.4″ N 17°31′31.5″ E), Grabowskie (54°06′15′ N 18°09′02′ E) (54°15′44.79″ N 18°15′58.54″ E), and Grabowko (54°15′09.47′ N 18°18′69.6′ E) (54°15′09.47″ N 18°18′69.6″ E). In the years 2016 and 2017, 50 mL samples were gathered from the investigated reservoirs by a qualified scuba diver. The samples were taken from the nearby shore as well as from the depths of 0 m, 5 m, 10 m, 15 m, and 20 m. In addition, one slime sample was also acquired at the depth of 20 m. The samples were collected in such a way as to avoid mixing of waters originating from different depths. Due to the isolation of pectinolytic bacteria from Jelen Lake,

the procedure for water sample collection from this reservoir was repeated every two months. Finally, this lake was sampled 6 times.

2.2. Isolation and Identification of Pectinolytic Strains

One hundred microliters of each water sample were plated on a semi-selective crystal violet pectate (CVP) medium [37] and incubated at 28 °C for 48 h. Bacterial colonies forming characteristic cavities were picked up and repeatedly plated in a reductive manner on CVP and tryptone soya agar (TSA) (Oxoid, Basingstoke, UK) as many times as it was necessary to reach the axenic culture state. All axenic pectinolytic isolates were subsequently frozen in 40% glycerol and kept at −80 °C for further analyses.

In order to assign pectinolytic isolates to the proper taxonomic groups, the bacterial biomass of the acquired strain was collected from the frozen stock and streaked in a reductive manner on TSA. After 24 h incubation at 28 °C, a single bacterial colony was suspended in 200 µL of sterile distilled water and frozen for 45 min at −20 °C and then defrosted. This procedure allows for effective lysis of the bacterial cells. Two microliters of such lysate were subsequently subjected to PCR-based identification.

At first, it was investigated whether the stated bacterial isolate belonged to the highly heterogeneous *P. carotovorum* group. This assignment was conducted by a multiplex PCR assay [38]. This reaction was carried out in a 25 µL PCR mixture containing 2 µL of the tested bacterial lysate, 1× reaction buffer supplemented with KCl (Thermo Fisher Scientific, Minneapolis, MN, USA), 2.5 mM $MgCl_2$, 80 µM of each dNTPs, 0.32 µM Df and Dr primers (for *Dickeya* genus), 0.1 µM Y45 and Y46 primers (for *P. atrosepticum*), 1.2 µM ExpccF and ExpccR primers (for bacteria formerly classified as *P. carotovorum*) in addition to 1 U of the *Taq* recombinant DNA Polymerase (Thermo Fisher Scientific, Minneapolis, MN, USA). The amplification was carried out with the use of the Thermal Cycler C1000 75 (Bio-Rad, Hercules, CA, USA) according to the following procedure: initial denaturation (95 °C, 4 min), 30 cycles of denaturation (94 °C, 45 s), annealing (62 °C, 90 s), and extension (72 °C, 90 s), with a final single extension step (72 °C, 3 min). Electrophoretic separation of the resulting amplicons was conducted in 1.5% agarose gel in 0.5× tris–borate–EDTA buffer at 100 V for 40 min. A 100 bp DNA molecular ladder (Thermo Fisher Scientific, Minneapolis, MN, USA) was used for the assessment of the length of the resultant PCR amplicons. After, 5 mg mL^{-1} ethidium bromide was utilized for staining. Then, the visualization process was performed under UV light in ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA). The isolates that gave specific PCR product of 535 bp with ExpccF and ExpccR primers were classified to the *P. carotovorum* species, and *P. carotovorum* SCRI 136 was used as a reference strain (Table 1).

Afterwards, we evaluated whether a strain classified to the *P. carotovorum* group should be assigned to *P. brasiliense* (formerly *P. carotovorum* subsp. *brasiliense*) species. Therefore, a single PCR assay was applied as described by Duarte et al. [16]. This PCR reaction was also performed in a total volume of 25 µL consisting of 2 µL of bacterial lysate and 1× PCR buffer supplemented with KCl, 2.5 mM $MgCl_2$, 0.1 mM of dNTPs, 1 µM BR1f and L1r primers, 1 U *Taq* recombinant DNA Polymerase. DNA amplification was performed under the following conditions: initial denaturation (94 °C, 2 min), 25 cycles of denaturation (94 °C, 45 s), annealing (62 °C, 45 s), and extension (72 °C, 90 s) with a final single extension step (72 °C, 10 min). Electrophoretic separation was carried out as mentioned above.

The further to-species identification of the acquired pectinolytic isolate was based on phylogenetic analysis and genomic profiling as described below.

Table 1. Strains of pectinolytic bacteria used in this study.

Species	Strain [a]	Source, Country of Isolation	Year of Isolation	Reference
Pectobacterium atrosepticum	SCRI 1086 IFB5103	*Solanum tuberosum*, Canada	1985	[39]
Pectobacterium carotovorum	SCRI 136 IFB5118	*Solanum tuberosum*, USA	NA	[40]
	IFB5369	*Solanum tuberosum*, Poland	2011	[41]
Pectobacterium brasiliense	CFBP 6617 LMG 21371 IFB5390	*Solanum tuberosum*, Brazil	1999	[16]
	HAFL05 IFB5527	*Solanum tuberosum*, Switzerland	2013	[42]
Pectobacterium parmentieri	SCC3193 IFB5308	*Solanum tuberosum*, Finland	1980s	[43]
Pseudomonas aeruginosa	ATCC 15692 PAO1 IFB9036	infected wound, Australia	1955	[44]
Pectobacterium aquaticum	CFBP 8637^T NCPPB 4640 A212-S19-A16	fresh water, France	2016	[35]
	CFBP 8636 A127-S21-F16		2015	
	CFBP 8632 A35-S23-M15		2015	
	CFBP 8633 A101-S19-F16		2016	
	CFBP 8634 A104-S21-F16		2015	
	CFBP 8635 A105-S21-F16		2016	
	IFB5637	fresh water, Poland	2016	this study

NA—not available. [a] IFB—Intercollegiate Faculty of Biotechnology University of Gdansk and Medical University of Gdansk, Poland; SCRI—The James Hutton Institute, bacterial collection, Scotland; CFBP—Plant–Associated Bacteria Collection, France; SCC—World Federation for Culture Collections, Netherlands; ATCC—American Type Culture Collection, USA; HAFL05—School of Agricultural, Forest, and Food Sciences HAFL, Switzerland; LMG—Belgian Coordinated Collections of Microorganisms (BCCM), Belgium; NCPPB—National Collection of Plant Pathogenic Bacteria, France.

2.3. Phylogeny and Genomic Profiling-Based Identification of IFB5637

Genomic DNA of the obtained pectinolytic isolate and reference strains (Table 1) was extracted from an overnight culture in tryptone soya broth (TSB) (BTL, Łodz,.Poland) of the stated strain using a commercially available Genomic Mini AX Bacterial Kit (A&A Biotechnology, Łodz,.Poland). The quality and concentration of the isolated DNA was assessed by using a NanoDrop ND-1000 (Thermo Fisher Scientific, Minneapolis, MN, USA).

The reassignment of the acquired pectinolytic isolate from *P. brasiliense* to *P. aquaticum* species was based on the comparison of the sequences of *dnaX* [45] and *recA* [46] genes. The isolated DNA of *P. aquaticum* IFB5637 and the reference strains (Table 1) was diluted to the concentration of 10 ng μL^{-1}. [36]. In the case of *dnaX*, PCR was performed in a 50 µL final solution mixture containing 2 µL of the previously diluted bacterial DNA, 1× PCR buffer supplemented with KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.4 µM of dnaXF and dnaXR primers, and 1 U *Taq* DNA Polymerase (Thermo Fisher Scientific, Minneapolis, MN, USA). The amplification was conducted using the following protocol: initial denaturation (94 °C, 3 min), 35 cycles of denaturation (94 °C, 1 min), annealing

(59 °C, 1 min), and extension (72 °C, 2 min) with a final single extension step (72 °C, 5 min). In the case of *recA*, PCR was performed in the 25 µL final solution mixture containing 1 µL of the previously diluted bacterial DNA, 1× PCR buffer supplemented with KCl, 0.25 mM $MgCl_2$, 0.13 mM of each dNTPs, 1 µM of recA1 and recA2 primers, and 1 U *Taq* recombinant DNA Polymerase. The amplification was conducted using the following protocol: initial denaturation (95 °C, 5 min), 32 cycles of denaturation (94 °C, 1 min), annealing (47 °C, 1 min), and extension (72 °C, 2 min) with a final single extension step (72 °C, 5 min).

The resultant PCR products of 535 bp for *dnaX* and 730 bp for *recA* were sequenced from both ends by a commercial company (Genomed, Warsaw, Poland). The chromatographic data were manually edited and aligned using the CLC Main Workbench version 6.9.1 (CLC Inc., Aarhus, Denmark) with default parameters. The edited sequences of *dnaX* and *recA* genes of the isolated strain were compared to *dnaX* and *recA* gene sequences available in the GenBank database. Concerning *dnaX* based phylogenetic tree reposted in this work, the following sequence was used GCA_003382645.2 for CFBP 8632; GCA_003382585.2 for CFBP 8635; GCA_003382645.2 for CFBP 8633; GCA_003382655.2 for CFBP 8636; GCA_003382595.2 for CFBP 8634 and TMK516879.1 for CFBP 8637TS. In addition, the sequences for *P. brasiliense* CFBP 6617 (MK516956.1), *P. carotovorum* (MW657239 for IFB5639; MK516909.1 for CFBP 2046), *P. atrosepticum* (BX950851.1 for SCRI 1046; MK516904.1 for CFBP 1526), *P. parmentieri* (CP003415.1 for SCC3193; MK516972.1 for CFBP 8475), and *D. dadantii* CFBP1269 (JX434940.1) were used for constructing a phylogenetic tree in MEGA X (Pennsylvania State University; www.megasoftware.net (accessed on 9 May 2021)) [47]. The tree was generated with a neighbor-joining algorithm with the Jukes–Cantor nucleotide distance measure. Bootstrap was set on the level of 1000 replicates.

Genomic profiling of the acquired pectinolytic isolate, named IFB5637, and the *P. aquaticum* reference strains (Table 1) were based on repetitive sequence PCR (rep-PCR). Rep-PCR reaction was conducted with ERIC primers according to Versalovic et al. [48]. The isolated DNA of *P. aquaticum* IFB5637 *P. aquaticum* and reference strains (Table 1) was diluted to the concentration of 10 ng μL^{-1} [36]. PCR was performed in a 25 µL final solution containing 5 µL of the diluted bacterial DNA, 1 × PCR buffer supplemented with $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, 0.1 mM of each dNTPs, 0.4 µM of ERIC1F and ERIC2R primers, and 2 U *Taq* recombinant DNA Polymerase. Amplification was conducted using the following protocol: initial denaturation (95 °C, 7 min), 30 cycles of denaturation (94 °C, 1 min), annealing (53 °C, 1 min), and extension (65 °C, 8 min) with a final single extension step (65 °C, 16 min). Electrophoretic separation of the resulting amplicons was conducted in 1% agarose gel in 0.5 × tris–borate–EDTA buffer at 50 V for 4 h. The outcomes were visualized in the same way as in the case of single PCRs as described above. A 1 kb DNA molecular ladder was used (Thermo Fisher Scientific, Minneapolis, MN, USA).

2.4. Phenotypic Characterization of the Collected IFB5637

Overnight bacterial cultures on TSA plates were prepared from the frozen socks of the collected IFB5637 isolate and the reference strains (Table 1). One bacterial colony was picked from each TSA plate and used for inoculation of the 5 mL TSB medium. Twenty-four hour incubation at 28 °C with 120 rpm shaking followed. These cultures were subsequently centrifuged (6000 rpm for 10 min), and the bacterial pellets were washed twice in 0.85% NaCl to remove any media residuals. Bacterial suspensions of OD_{600} = 0.1 (approximately 10^8 CFU mL^{-1}) in 0.85% NaCl solution were prepared for IFB5637 and the reference strains (Table 1) and used in the following procedures if not stated otherwise.

2.4.1. Pathogenicity Assays

The ability of IFB5637 isolate to cause disease symptoms was evaluated on potato slices and chicory leaves as described previously by Zoledowska et al. [11] and Van Gijsegem et al. [49], respectively.

Regarding the potato slices maceration assay, potato tubers cv. Lord were washed under tap water and sterilized with 10% sodium hypochlorite solution for 20 min. Later,

the tubers were air-dried under laminar flow cabinet and then aseptically cut into 10 mm thin slices. Subsequently, in each slice 5 mm diameter holes were drilled. Each hole in every potato slice was inoculated with 50 µL of OD_{600} = 0.1 bacterial suspension. The slices were placed on wet linen in plastic boxes covered with lids for assuring high relative humidity and incubated at 28 °C for 48 h. Diameters of the rotten spots were measured afterwards. As a negative control, 0.85% NaCl was used. Three biological repetitions of this experiment, each with nine technical replications, were carried out.

Concerning pathogenicity on chicory leaves, sterile pipette tips were used for drilling leaves at the base of each chicory leaf. The leaves were inoculated with 10 µL of OD_{600} = 0.1 bacterial suspension and incubated in plastic bags at 28 °C for 48 h in high relative humidity. Subsequently, the length of the rotten tissue was measured. Three biological repetitions of the experiment, involving ten chicory leaves per strain, were performed.

2.4.2. Activity of Plant-Cell-Wall-Degrading Enzymes

Bacterial ability to produce plant-cell-wall-degrading enzymes, including pectinases, cellulases, lipases, and proteases, was assessed according to the previously described protocols [50–53].

The activity of pectinases was tested on a M63 + 0.25% poligalacturonate (PGA) medium [50]. Two microliters of the OD_{600} = 0.1 bacterial suspension were spotted on this plate and incubated at 28 °C for 48 h. An aqueous 10% copper acetate solution was subsequently applied on the surface of the medium for 10 min to visualize PGA degradation zones. The resultant halo zones around bacterial colonies were measured.

The activity of cellulases was evaluated on a M63 + 1% carboxymethylcellulose (CMC) medium [51]. Two microliters of the OD_{600} = 0.1 bacterial suspension were spotted on this plate and incubated at 28 °C for 48 h. Afterwards, the medium was stained with 1% Congo red dye solution for 5 min and washed two times with 4 M NaCl solution. Brown halo zones visible around the colonies, which corresponded to the exhibited cellulase activity, were measured.

Lipase activity was analysed with the use of a Rhodamine medium [52]. Two microliters of the OD_{600} = 0.1 bacterial suspension were spotted on this plate and incubated at 28 °C for 48 h. The clear halos observed around bacterial colonies indicating lipase activity were measured.

Proteases activity was tested on skim milk agar medium [53]. Two microliters of the OD_{600} = 0.1 bacterial suspension were spotted on this plate and incubated at 28 °C for 48 h. The protease activity caused degradation of casein in the medium resulting in clear halo zones around the colonies which were measured.

The described experiments were performed in three biological repetitions with four technical repetitions each.

2.4.3. Biochemical Profile

Biochemical features of the IFB5637 isolate were determined with the use of the API 20E (Biomérieux, Craponne, France) commercial assay including the following tests: ONPG (β-galactosidase production), CIT (utilization of citrate), VP (production of acetoin), GEL (gelatinase production), ADH (arginine dihydrolase), LDC (lysine decarboxylase), ODC (ornithine decarboxylase), H_2S production, URE (urease production), TDA (utilization of tartarate), IND (indole production), VP (acetoin production), GEL (gelatinase production), GLU (glucose fermentation), MAN (mannitol fermentation), INO (inositol fermentation), SOR (sorbitol fermentation), RHA (rhamnose fermentation), SAC (saccharose fermentation), MEL (melibiose fermentation), AMY (amygdalin fermentation), and ARA (arabinose fermentation). The previously prepared OD_{600} = 0.1 bacterial suspension was used for inoculation of the API microtubes prior to 24 h incubation at 28 °C. The results were collected and interpreted according to the provided manufacturer's instructions.

2.4.4. Other Factors Involved in Virulence

Bacterial ability to secrete siderophores was determined on chrome azurol S-agar plates [54]. Two microliters of the OD_{600} = 0.1 bacterial suspension were spotted on this plate and incubated at 28 °C for 48 h. The orange halo zones that formed around the colonies were measured. This experiment was carried out in three biological repetitions with four technical replications each.

Motility assays were performed on TSA medium solidified either with 0.3% agar or 0.6% agar, regarding swimming or swarming [55], respectively. Two microliters of the OD_{600} = 0.1 bacterial suspension were spotted on these plates and incubated at 28 °C for 24 h. The diameters of the bacterial colonies were measured after 24 h. Each test was carried out in three biological repetitions, each involving four technical ones.

Salt tolerance was assayed on TSA medium with 5% NaCl [56]. Two microliters of the OD_{600} = 0.1 bacterial suspension were spotted on this plate and incubated at 28 °C for 48 h. Salt tolerance was stated if bacterial growth was observed on such a plate.

Bacterial ability to form biofilm was determined as described by Nykyri et al. [57]. In more detail, 10 µL of an overnight bacterial culture in TSB was added to Eppendorf tubes containing 400 µL of M9 minimal medium [58]. After 16 h of static incubation at 18 °C, bacterial biofilm formed on the walls of the tubes. For staining, 70 µL of 1% crystal violet solution was added into each tube and incubated at room temperature for 20 min. The tubes were washed with distilled water and air-dried. Subsequently, 600 µL of 96% ethanol was utilized to extract the absorbed crystal violet dye. One hundred microliters of the resultant suspension were placed in a 96-well microtest plate and quantified spectrophotometrically at 565 nm with a Victor2 I420 Multilabel Counter (Wallac, Finland).

Isolation and determination of the sugar composition of the O-polysaccharide (OPS) of bacterial lipopolysaccharide (LPS) was performed as described by Kowalczyk et al. [59]. The analyzed strain was plated from frozen stock and cultured on TSA medium at 28 °C for 24 h. Then, 200 mL TSB medium was inoculated with a single bacterial colony taken from the TSA plate and subsequently incubated at 28 °C with 130 rpm shaking for 24 h. This overnight culture was used for inoculation of 2 L of TSB medium. Incubation for 48 h at 28 °C with 130 rpm shaking followed. Bacterial cells were harvested using the centrifuge 6-16KS (Sigma, Kanagawa, Japan) at 16,915.34× *g* for 10 min and stored at −20 °C. The above-described procedure was repeated until 50 g of the IFB5637 strain biomass was collected. The LPS was isolated from dried bacterial cells using hot phenol extraction and purified by enzymatic digestion (DNAse, RNAse, and Proteinase K). The OPS was obtained after hydrolysis of the LPS with acetic acid and separation by size exclusion chromatography. Finally, sugar analysis (hydrolysis with trifluoroacetic acid, reduction with sodium borohydride, acetylation in acetic anhydride, and analysis by gas chromatography-mass spectrometry technique) was performed to identify and determine the number of monosaccharide residues in the O-polysaccharide.

2.5. Statistical Analysis

Statistical analysis of phenotypic data was conducted using R, version 3.3.2 (31 October 2016) (R Core Team 2014, Vienna, Austria). Levene's test was applied for testing the equality of variances and the Shapiro–Wilk's evaluation was implemented for evaluating normality of the results. Depending on the outcomes of the above-listed tests, either ANOVA followed by Tukey's honest significance test or Kruskal–Wallis test together with a post-hoc analysis applying Fisher's least significance criterion were utilized. $p < 0.05$ was applied in all the calculations.

3. Results and Discussion

The *P. aquaticum* IFB5637 strain was isolated from water samples collected on (2 October 2016) from Jelen Lake from 0 m depth nearby the shore. Jelen Lake is a kettle lake located in the Polish region of Kashubia. According to a 2006 report, this lake was in the 1st class in terms of water purity, i.e., it exhibited a low level of organic matter, minimal numbers of biogenic substances in addition to low concentrations of dissolved inorganic matter [60]. Of the

parameters analyzed, only the elevated level of total nitrogen was confirmed [60]. When the water was sampled, the air temperature was 17 °C and the water temperature was 16 °C. As the obtained *P. aquaticum* IFB5637 isolate was the first strain from this species isolated from the waterways in Poland, we performed its detailed characterization.

3.1. Genotypic Features of *P. aquaticum* IFB5637 Strain

The BLAST comparison of the fragments of gene sequences of *dnaX* and *recA* of the identified isolate and the sequences of *Pectobacteriaceae* strains deposited in the GenBank database showed 99.38% and 97.38% identity in addition to 100% and 93% coverage, respectively, between the *dnaX* and *recA* sequences of the acquired pectinolytic isolate *P. aquaticum* IFB5637 and the corresponding sequences of *P. aquaticum* CFBP 8637^T (accession numbers TMK516879.1 and MK517167.1) [35]. The sequences of *dnaX* and *recA* genes of *P. aquaticum* IFB5637 were submitted to GenBank and are available under the following accession numbers: MW657238 and MW660584. Regarding single-nucleotide polymorphisms (SNPs) detected in the 535-bp *dnaX* gene fragments of *P. aquaticum* isolates (IFB5637, CFBP 8632, CFBP 8636, CFBP 8633, CFBP 8637^T, CFBP 8636, CFBP 8634), we were able to distinguish two SNP regions (at 222 bp position and at 451 position) in which the C–T transition occurred in *P. aquaticum* IFB5637. The *recA* gene sequence of IFB5637 was identical to the sequence of CFBP 8636 strain and the other *P. aquaticum* (CFBP 8632, CFBP 8633, CFBP 8637^T, CFBP 8636, CFBP 8634).

A neighbor-joining tree, constructed on the sequences of the *dnaX* (Figure 1) and *recA* (data not shown) gene fragments of the tested isolate in comparison to the reference *Pectobacteriaceae*. (Table 1) strains, grouped the sequences of the studied isolate and the sequences of the reference strains of *P. aquaticum* into one distinct clade.

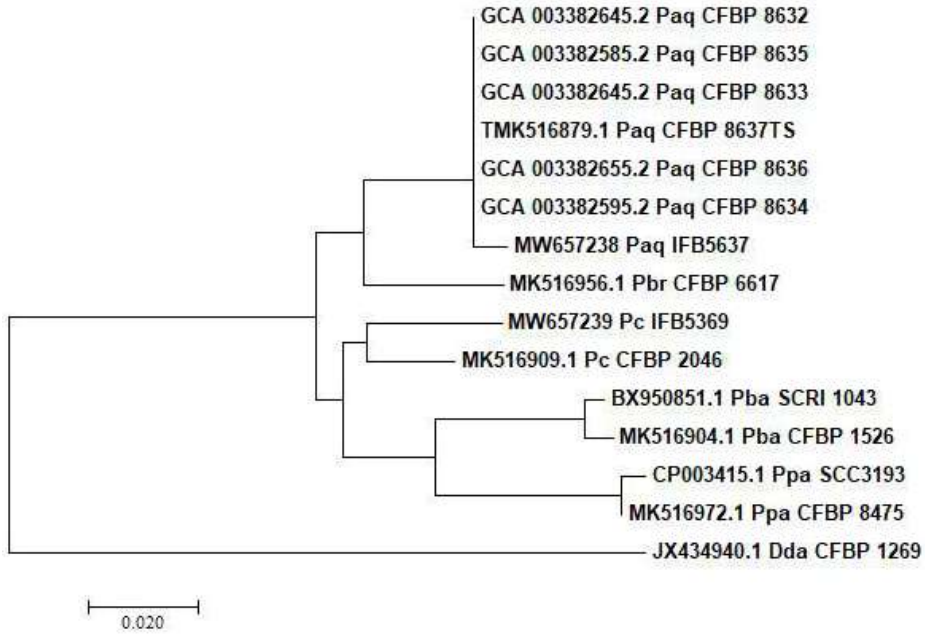

Figure 1. Phylogenetic analysis based on 535 bp fragment of *dnaX* gene. *P. aquaticum* IFB5637 isolated in Poland was juxtaposed to the *Pectobacteriaceae* reference strains. The sequences of the utilized strains: *P. aquaticum* (Paq CFBP 8632, Paq CFBP 8635, Paq CFBP 8633, Paq CFBP 8637^T, Paq CFBP 8636, Paq CFBP 8634, *P. brasiliense* (Pbr CFBP 6617), *P. carotovorum* (Pc CFBP 2046), *P. atrosepticum* (Pba SCRI 1043, Pba CFBP 1526), *P. parmentieri* (Ppa SCC3193, Ppa CFBP 8475), and *D. dadantii* (Dda CFBP 1269) were downloaded from the GenBank database (07.2020). The tree was generated with a neighbor-joining algorithm with the Jukes–Cantor nucleotide distance measure in the MEGA X software package. Bootstrap was set on the level of 1000 replicates.

The conducted genomic profiling with the use of ERIC primers (Figure 2), revealed small differences between the tested *P. aquaticum* IFB5637 and the reference strains of *P. aquaticum*, i.e., CFBP 8637^{T}, CFBP 8632, CFBP 8633, CFBP 8634, CFBP 8635, and CFBP 8636. The acquired ERIC patterns for CFBP 8636, CFBP 8632, and CFBP 8634 strains of *P. aquaticum* isolated in 2015 were less heterogenous than in the case of the other two *P. aquaticum* strains (CFBP 8633, CFBP 8635) from 2016. Similar results were presented in the publication by Zoledowska et al. [11], where differences in the profiles of *P. parmenteri* isolates obtained in Poland in different years were observed. However, the genomic profiles of *P. aquaticum* strains were not as homogeneous as in the case of *Dickeya solani* isolates, as presented in a study by Golanowska et al. [61]. In that research, REP genomic profiles of all tested *D. solani* strains from Poland, Finland, and Israel turned out to be exactly the same as the molecular pattern of *D. solani* IPO 2222^{T}.

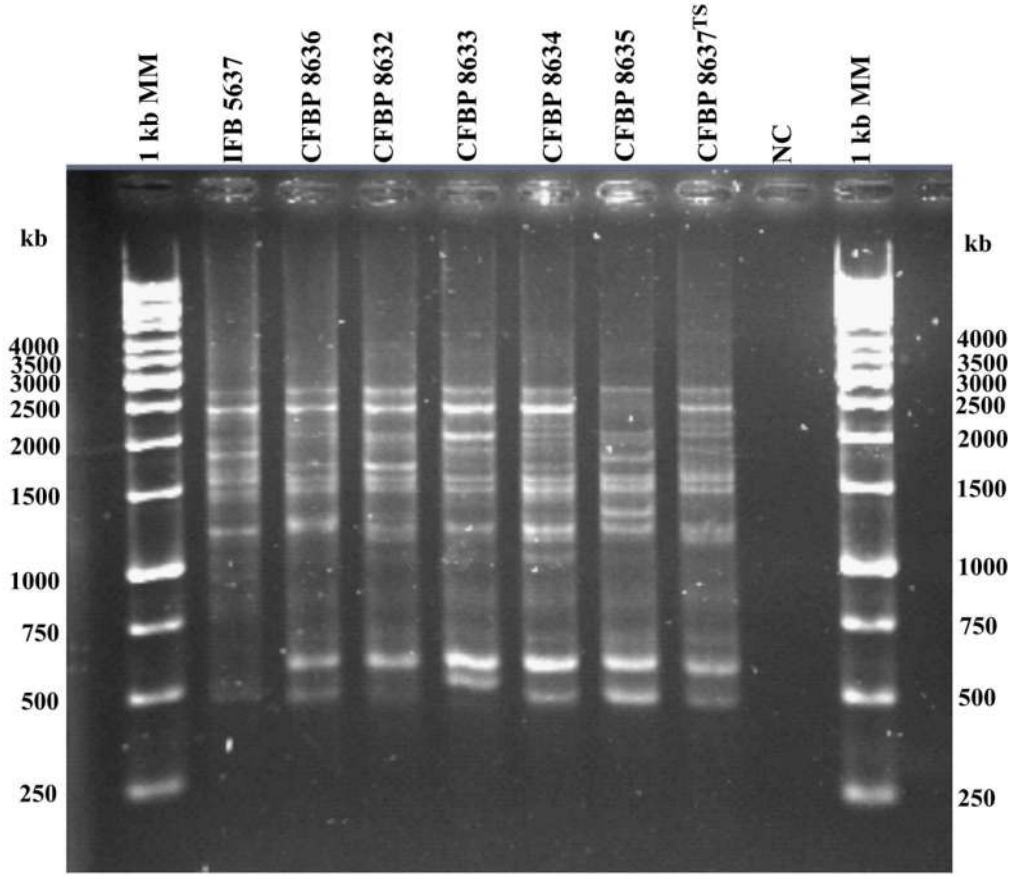

Figure 2. ERIC-based genomic profiling of *P. aquaticum* strains. MM—1-kb DNA Ladder (Thermo Fisher Scientific, Minneapolis, MN, USA); IFB5637—*P. aquaticum* isolated in the frames of this study; *P. aquaticum* reference strains: CFBP 8637^{T}, CFBP 8632, CFBP 8633, CFBP 8634, CFBP 8635, and CFBP 8636; NC—negative control.

3.2. Phenotypic Features of *P. aquaticum* IFB5637 Strain

The studied *P. aquaticum* IFB5637 of water origin showed an ability to macerate potato tubers and chicory leaves. In more detail, the analyzed isolate exhibited weaker virulence on potato and chicory in comparison to the two tested *P. brasiliense* (CFBP 6617 and HAFL05) and two *P. carotovorum* (SCRI 136 and IFB5637) strains (Figure 3). However, its ability to macerate plant tissue was higher than that of *P. atrosepticum* SCRI 1086. Moreover, in the

previous work [35], *P. aquaticum* strains isolated from water in France were shown to be capable of causing disease symptoms on potato tuber tissue.

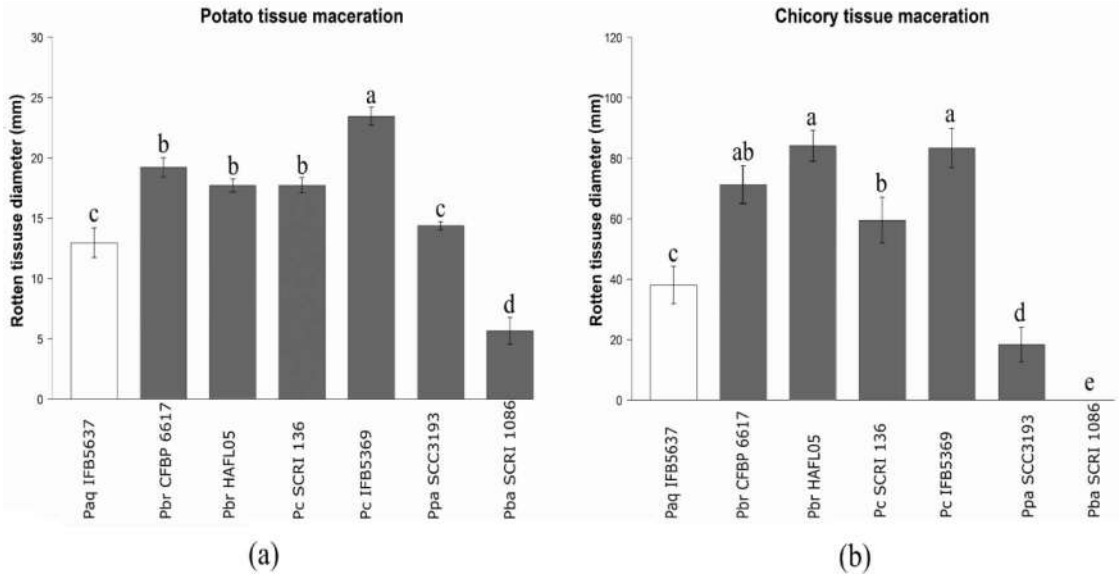

Figure 3. Comparison of the potato (**a**) and chicory (**b**) tissue maceration ability of the tested *P. aquaticum* IFB5637 strain isolated from water in Poland in contrast to the closely related *Pectobacteriaceae* strains. Strains abbreviations: *P. aquaticum* (Paq IFB5637), *P. brasiliense* (Pbr CFBP 6617, Pbr HAFL05), *P. carotovorum* (Pc SCRI 136, Pc IFB5369), *P. parmentieri* (Ppa SCC3193), and *P. atrosepticum* (Pba SCRI 1086). Means ± SE of diameters (potato) or lengths (chicory) of the rotten tissues are depicted. Three independent experiments with nine technical replications were conducted. Means marked with different letter (a, b, c, d, e) are significantly different according to the Kruskal–Wallis test followed by a post-hoc analysis applying Fisher's least significant criterion at $p < 0.05$.

The *P. aquaticum* IFB5637 strain turned out to be able to produce a wide spectrum of plant-cell-wall-degrading enzymes (Table 2). It is worth noticing that *P. aquaticum* IFB5637 showed a significantly lower pectinase and protease activities than the tested *P. brasiliense* (CFBP 6617 and HAFL05) and *P. carotovorum* strains (SCRI 136 and IFB5369). The low pectinases and proteases activities exhibited by the analyzed *P. aquaticum* IFB5637 (Table 2) were correlated with the low maceration capacity towards potato and chicory tissues. Similar outcomes were reported by Potrykus et al. [62] during the study of the closely related *D. solani* strains. In that work, the low virulent *D. solani* IFB0223 strain did not show any protease activity and significantly lower pectinase activity, in contrast to the highly virulent *D. solani* IFB0099 strain, which exhibited high pectinase and protease activities. Moreover, the above-listed *D. solani* strains differed in the ability to macerate plant tissue.

Concerning the capacity of *P. aquaticum* IFB5637 to chelate iron ions, we observed a relatively high potency of this strain to produce siderophores (Table 2). On the other hand, the reference strains *P. parmentieri* SCC3193 and *P. atrosepticum* SCRI 1086 revealed no siderophore production ability, which is consistent with the results obtained by Zoledowska et al. [11].

The motility assay indicated that *P. aquaticum* IFB5637 is capable of moving, either by swimming or swarming (Table 2). Interestingly, the *P. aquaticum* IFB5637 strain from water exhibited lower swarming and swimming capacities than the closely related *P. brasiliense* strains: CFBP 6617 and HAFL05 (Table 2). The obtained results are in agreement with the data shown in a study by Ozturk et al. [41], where the lowest swimming motility was presented by the *P. atrosepticum* isolates while the highest by *P. brasiliense* and *P. carotovorum* strains.

Table 2. Phenotypic characteristics of *P. aquaticum* IFB5637 strain isolated in Poland in comparison to the tested *Pectobacteriaceae* strains.

Activities	*P. aquaticum* IFB5637	*P. brasiliense* CFBP 6617	*P. brasiliense* HAFL05	*P. carotovorum* SCRI 136	*P. carotovorum* IFB5369	*P. parmentieri* SCC3193	*P. atrosepticum* SCRI 1086
Pectinases activity	11.0 ± 2.7	19.3 ± 1.2	22.0 ± 1.5	22.0 ± 1.7	22.3 ± 0.8	16.9 ± 1.7	10.8 ± 0.7
Proteases activity	6.3 ± 1.4	14.3 ± 1.5	16.4 ± 1.4	13.0 ± 1.4	19.7 ± 1.3	5.7 ± 1.2	2.9 ± 1.5
Cellulases activity	13.5 ± 0.8	6.0 ± 1.4	12.1 ± 0.7	11.7 ± 0.8	13.6 ± 0.9	0.0 ± 0.0	0.0 ± 0.0
Lipases activity	13.4 ± 0.5	16.8 ± 0.5	19.3 ± 0.6	17.5 ± 0.6	13.5 ± 0.9	15.3 ± 0.4	9.3 ± 0.5
Siderophores activity	7.1 ± 0.2	10.8 ± 0.3	2.8 ± 1.2	4.5 ± 1.0	7.8 ± 0.7	0.0 ± 0.0	0.0 ± 0.0
Swimming	22.8 ± 1.7	39.8 ± 3.2	41.6 ± 1.5	34.2 ± 1.6	39.4 ± 2.2	13.0 ± 0.7	5.2 ± 0.4
Swarming	4.0 ± 0.4	10.5 ± 4.1	9.3 ± 3.5	4.8 ± 0.7	14.4 ± 5.6	9.7 ± 3.6	4.1 ± 0.2
Biofilm formation *	+	+++	++	+	+	++	+

* The biofilm formation capacity of *P. aquaticum* IFB5637 was compared to that of *Pseudomonas aeruginosa* ATCC 15692 strain. The collected results are designated as follows: +++ strain was able to form biofilm as efficiently as *P. aeruginosa* ATCC 15,692; ++ strain was capable of forming biofilm significantly less efficiently than the reference strain; + marks the least potent biofilm producers.

Additionally, *P. aquaticum* IFB5637 exhibited a lower ability of biofilm formation (Table 2) than the other tested *Pectobacteriaceae* strains. It is worth noting that one of the tested strains of *Pectobacteriaceae* (*P. brasiliense* CFBP 6617) exhibited biofilm production at the same level as the *Pseudomonas aeruginosa* ATCC 15692 strain included as a positive control in this experiment. Besides, both intra-species variation and inter-species variation in the biofilm formation potency could be observed.

Based on the API 20E assay, it was found that the *P. aquaticum* IFB5637 strain was able to ferment mannitol, rhamnose, saccharose, amygdalin, and arabinose like all the other *Pectobacterium* spp. tested (Table 3). Similar results for the reference strains were described in the study by Ozturk et al. [41], which confirmed the ability of the analyzed strains of *P. atrosepticum*, *P. carotovorum* subsp. *brasiliense*, *P. carotovorum* subsp. *carotovorum*, and *P. parmentieri* to utilize L-rhamnose, D-mannitol, raffinose and L+arabinose. However, contrary to the investigated *Pectobacterium* spp. strains, *P. aquaticum* IFB5637 did not degrade melibiose (Table 3). The *P. aquaticum* IFB5637 strain was also stated to be capable of gelatinase production in contrast to the closely related *P. brasiliense* strains CFBP 6617 and HAFL05. Moreover, all the herein tested *Pectobacterium* spp. strains, including *P. aquaticum* IFB5637, were able to produce β-galactosidase and acetoin. The *P. aquaticum* IFB5637 strain was shown to be unable to utilize sorbitol and to grow on 5% NaCl (Table 3) similar to the *P. aquaticum* strains investigated by Pedron et al. [35].

The analysis of the chemical structure of *P. aquaticum* IFB5637 LPS revealed that the *P. aquaticum* IFB5637 strain produces a smooth form of LPS, which is known as one of the important virulence factors responsible for adherence of bacteria and plant tissue colonization. The OPS of *P. aquaticum* IFB5637 LPS turned out to contain three different monosaccharides, namely, mannose, glucose, and abequose (3,6-dideoxy-D-xylo-hexose). Mannose and glucose are relatively common monosaccharides in bacterial O-polysaccharides, while abequose has not been identified yet in any strains of the *Pectobacteriaceae* family [59,63,64].

As suggested by Pedron et al. [35], divergence within the *Pectobacterium* species outside the plant context is highly understudied. The herein presented data contribute to broadening the knowledge on the newly established *P. aquaticum* species. As far as we are aware, the herein presented research is the second study on genotypic and phenotypic features of this surface-water-associated pectinolytic species. We found that sequencing of *dnaX* [45] and *recA* [46] genes could be used for distinction of *P. aquaticum* species. Moreover, in view of the unavailability of *P. aquaticum*-specific PCR reactions, Pedron et al. [35] observed that the sequencing of *gapA*, in contrast to 16S rRNA, is informative enough for differentiation of *P. aquaticum* strains. In accordance with high dDDH and pairwise ANI values, equaling >86% and ≥98%, respectively, as calculated by Pedron et al. [35], we herein observed rather small differences in the ERIC-based genomic profiles of all the previously described *P. aquaticum* strains (Figure 2). We also reportedthe API 20E biochemical profile of Paq IFB5637; however, it did not discriminate this strain from, for example, *P. atrosepticum* SCRI 1086. Therefore, API 20E profiling is not distinctive enough in contrast to Omnilog-based identification of Pedron et al. [35] involving, for instance,

metabolic reactions with trehalose, cellobiose or formic acid that differentiated *P. aquaticum* from the other tested *Pectobacterium* spp. We observed that *P. aquaticum* IFB5637 was able to efficiently macerate potato slices and chicory leaves; this is in accordance with Pedron et al. [35], who reported the maceration capacity of *P. aquaticum* strains on potato tubers under laboratory conditions. Notably, we studied for the first time in *P. aquaticum* the activities of plant-cell-wall-degrading enzymes, which are the most important virulence factors of soft rot *Pectobacteriaceae*. Even if the noted action of cellulases and proteases was lower than, for example, that of highly virulent *P. brasiliense* strains, it was higher than the one attributed to the included *P. atrosepticum* strain, isolated from the rotted potato tubers. *P. aquaticum* IFB5637 also showed significant production of siderophores and high swimming motility which further supports its virulence potential. Importantly, we provided the first insights into the sugar composition of O-polysaccharide of *P. aquaticum* LPS. LPS molecules are associated with the virulence of bacterial strains, especially during colonization and overcoming of the defense mechanisms of the plant host. The herein presented data suggest that the usage of surface waters containing pectinolytic bacteria, even if the members of a certain taxonomic group have not been previously isolated from the affected plant tissue, for the irrigation of potato fields can potentially contribute to the spread of soft rot and blackleg diseases.

Table 3. Biochemical features of the *P. aquaticum* IFB5637 strain in comparison to the other tested *Pectobacteriaceae* strains.

Biochemical Features	*P. aquaticum* IFB5637 ^{T}	*P. brasiliense* CFBP 6617	*P. brasiliense* HAFL05	*P. carotovorum* SCRI 136	*P. carotovorum* IFB5369	*P. parmentieri* SCC3193	*P. atrosepticum* SCRI 1086
ß-galactosidase production	+	+	+	+	+	+	+
Arginine dihydrolase production	−	+	−	−	+	−	−
Lysine decarboxylase production	−	−	−	−	−	−	−
Ornithine decarboxylase production	−	−	−	−	−	−	−
Utilization of citrate	+	+	+	+	+	−	+
H_2S production	−	−	−	−	−	−	−
Urease production	−	−	−	−	−	−	−
Utilization of tartrate	+	+	+	+	+	+	+
Indole production	−	−	+	−	−	−	−
Production of acetoin	+	+	+	+	+	+	+
Gelatinase production	−	+	+	−	+	−	−
Glucose fermentation	−	−	−	−	−	−	−
Mannitol fermentation	+	+	+	+	+	+	+
Inositol fermentation	−	−	−	−	−	−	−
Sorbitol fermentation	−	−	−	−	−	−	−
Rhamnose fermentation	+	+	+	+	+	+	+
Saccharose fermentation	+	+	+	+	+	+	+
Melibiose fermentation	−	+	+	+	+	+	−
Amygdalin fermentation	+	+	+	+	+	+	+
Arabinose fermentation	+	+	+	+	+	+	+
Growth in 5% NaCl	−	+	+	−	+	−	−

+ Refers to possession of a specific metabolic feature, − lack of such a trait.

4. Conclusions

To the best of our knowledge, this is the first report on the isolation of the *P. aquaticum* strain in Poland. *P. aquaticum* IFB5637 acquired from Jelen Lake was capable of macerating potato and chicory tissues. This strain exhibited the activity of plant-cell-wall-degrading enzymes that are important virulence factors. The study on the OPS of the LPS of *P. aquaticum* revealed the presence of common sugars, such as mannose and glucose in addition to abequose, identified for the first time among the *Pectobacteriaceae* family. The presented data suggest that the usage of surface waters for irrigation of potato fields can potentially contribute to soft rot bacteria spread.

Author Contributions: W.B. collected water samples, performed all microbiological experiments, and visualized the collected data. A.M.-P., W.S., and E.L. took part in discussion on the collected data. A.K. and Z.K. performed the analysis of the LPS structure. W.B. wrote the first version of this manuscript. W.B., A.M.-P., and E.L. prepared the final version of this manuscript that was accepted by all authors. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Determined sequences data of *dnaX* and *recA* genes of *Pectobacterium aquaticum* IFB5637 were deposited in GenBank under the following accession number MW657238 (*dnaX* gene) and MW660584 (*recA* gene).

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ANOVA—analysis of variance; API—analytical profile index; CVP—crystal violet pectate; LPS—lipopolysaccharides; OPS—O-polysaccharide; SNPs—single-nucleotide polymorphisms; TSA— tryptone soya agar; TSB—tryptone soya broth; PCWDEs—plant-cell-wall-degrading enzymes; Rep-PCR—repetitive sequence PCR.

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AUTHOR CONTRIBUTION STATEMENT

I declare that in the manuscript:

"The first polish isolate of a novel species *Pectobacterium aquaticum* originates from a pomeranian lake"

Weronika Babińska, Agata Motyka-Pomagruk, Wojciech Śledź, Agnieszka Kowalczyk, Zbigniew Kaczyński, Ewa Łojkowska

my contribution involved conceived and designed all experiments. Moreover I collected water samples, performed all microbiological experiments, preparation of bacterial biomass for the analysis of the LPS structure and visualized the collected data. I took part in the discussion on the collected data. I prepared first version of this manuscript and corrected it according to the co-authors' suggestions.

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my involvement included participation in planning of the described experiments, corrections of the first version of the manuscript and discussion of the research results.

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my contribution involved conceived and designed all experiments. Moreover, I took part in the preparation of bacterial biomass for the analysis of the LPS structure.

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my contribution involved conceived and designed experiment for the analysis of the OPS structure. Moreover I performed the analysis of the OPS structure. Finally I took part in the discussion on the collected data and provided correction to the first version of the manuscript.

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my contribution consisted of planning studies on the sugar composition of the OPS. I also participated in the discussion of the collected data and made corrections to the first version of the manuscript.

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I declare that, I was responsible for co-conceptualization of the project and participation in the discussion of the results as well as work on final version of the manuscript.

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The structure of an abequose - containing O-polysaccharide isolated from *Pectobacterium aquaticum* IFB5637

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The structure of an abequose - containing O-polysaccharide isolated from *Pectobacterium aquaticum* IFB5637

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ABSTRACT

Soft rot and blackleg diseases, caused by pectinolytic bacteria from the numerous species of *Dickeya* and *Pectobacterium*, pose a serious threat to the world potato production. Besides, infections triggered by these pectinolytic bacteria lead to huge economic losses in the cultivation of other crops, vegetables, and ornamentals. Strains belonging to the genus *Pectobacterium* tend to be isolated from various environments such as rotten or asymptomatic plants, weeds, soil or water. The main virulence factors of these phytopathogenic bacteria involve plant cell wall degrading enzymes (PCWDEs) *i.e.* pectinases, cellulases and proteases. Among accessory virulence factors, there is often lipopolysaccharide (LPS) listed. This constituent of the external part of bacterial cell wall contains lipid A, inner and outer core in addition to O-polysaccharide (OPS). LPS plays an important role in plant-microbe interactions, in particular during the first step of pathogen recognition. In this study we present the chemical structure of OPS of the first *Pectobacterium aquaticum* strain (IFB5637) isolated from water in Poland. The OPS consists of two common hexoses, such as mannose and glucose, as well as an abequose (3,6-dideoxy-D-*xylo*-hexose), the first 3,6-dideoxyhexose identified among the *Pectobacteriaceae* family:

α-Abe*p*
↓3
β-D-Man*p*
↓4
→3)-β-D-Glc*p*-(1→2)-α-D-Man*p*-(1→3)-α-D-Glc*p*-(1→

According to our best knowledge this is the first determined structure of the OPS of *P. aquaticum*.

1. Introduction

Pectinolytic bacteria from the genera *Pectobacterium* and *Dickeya* belonging to the *Pectobacteriaceae* family cause blackleg and soft rot diseases on potato and soft rot on a wide range of plants, including important crops, vegetables and ornamentals [1]. The strains from the genus *Pectobacterium* tend to be isolated not only from infected, symptomatic or asymptomatic plants, but also from soil or water. The species *Pectobacterium aquaticum* was established by Pedron at al. after isolation and characterisation of the strains originating from different waterways of South-Eastern France [2]. *P. aquaticum* IFB5637 is the first strain belonging to this species that has been isolated from a lake in Poland [3]. Previous studies [2,3] indicated that *P. aquaticum* was able to produce a broad spectrum of virulence factors including plant cell wall degrading enzymes (PCWDEs), such as pectinases, cellulases and proteases. Despite the fact that *P. aquaticum* strains have been collected from aquatic

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environments, these bacteria are capable of macerating potato and chicory tissues under laboratory conditions [2,3].

Among the accessory virulence factors of soft rot *Pectobacteriaceae* (SRP), an external constituent of the bacterial cell wall, namely lipopolysaccharide (LPS), is often listed. This molecule consists of lipid A, inner and outer core in addition to O-polysaccharide (OPS) and plays a role in plant-microbe interactions, in particular during the first step of infection involving recognition of the pathogen. In the current research, we aimed at elucidating the chemical structure of OPS of *P. aquaticum* IFB5637 and conducting a comparative study with the earlier identified OPS molecules of the other members of the *Pectobacterium* genus.

2. Results

In order to determine the chemical structure of O-polysaccharide of *P. aquaticum* IFB5637 [3], the LPS was isolated from bacterial cells using two types of extractions: PCP (Phenol-Chloroform-Petroleum ether) [4] and hot phenol-water extraction [5], then purified by enzymatic digestion. The extracted LPS was hydrolyzed to separate the lipid A from the sugar part. Lipid A was centrifuged, and the sugar portion was separated by size-exclusion chromatography (SEC). Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded for the obtained fractions. Based on them, a fraction containing pure polysaccharide was selected and used for further structural studies.

The obtained OPS was characterized by chemical methods. The sugar analysis was used to identify monosaccharides and to determine their number in the repeating unit of the OPS. The gas-liquid chromatography-mass spectrometry (GLC-MS) analysis of the obtained derivatives showed the presence of two different hexoses in the molar ratio ~1:1. The hexoses were identified as mannose and glucose by gas-liquid chromatography (GLC) analysis of the alditol acetates derivatives with the corresponding standards. The absolute configuration of the monosaccharides was established by GLC analysis of the synthesized (*R*,*S*)- and (*S*)-2-butyl-glycosides of sugar residues of the OPS and the respective standards. The absolute configuration D was determined for both Glc and Man. The results of methylation analyses revealed the presence of three partially methylated alditol acetate derivatives: two different 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol and 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methylhexitol in the approximate molar ratio 2 : 1: 1. Taking into account the results of the sugar analysis, the presence of 2x →3)-Glc, →3)-Man and →2,4)-Man residues or 2x →3)-Man, →3)-Glc and →2,4)-Glc residues can be assumed. Wondering at this stage of the analysis was the presence of a branching sugar residue and the absence of a terminal sugar residue.

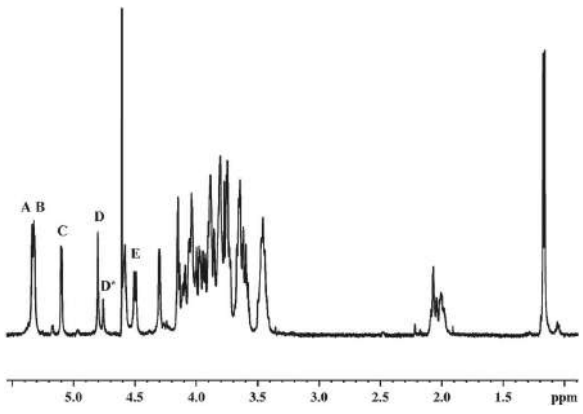


Fig. 1. ^{1}H NMR spectrum of the OPS isolated from *Pectobacterium aquaticum* IFB5637. The letters refer to the monosaccharides residues as defined in Table 1.

The next step in the structural analysis was the interpretation of the recorded NMR spectra to identify the spin systems and to determine the number, the ring size, the α or β anomeric configuration, the position of the substitutions and the sequence of the sugar residues in the repeating unit of the OPS [6]. The analysis of the ^{1}H NMR spectrum (Fig. 1) and HSQC (heteronuclear single quantum coherence) spectrum (Fig. 1S) in the characteristic range of the anomeric proton spectrum clearly showed that the O-antigen of the bacterium *P. aquaticum* IFB5637 is composed of five sugar units. This observation was inconsistent with the results of chemical analysis, which revealed the presence of only four sugar residues. The inaccuracy was clarified during the subsequent steps of NMR spectra analysis. The signals labeled **A-E** represent five anomeric protons at δ: 5.333 (**A**), 5.319 (**B**), 5.097 (**C**), 4.797 (**D**) and 4.493 (**E**) in the estimated molar ratio 1:1:1:1:1. The proton spectrum contains also a characteristic signal of methyl group of 6-deoxy monosaccharide at δ ~1.2, a signal of –CH_2 group (probably) at δ ~2.0 and the remaining protons signals in the region of δ 3.4–4.3. The analysis of one-dimensional ^{1}H NMR spectrum together with two-dimensional NMR spectra [homonuclear COSY (correlation spectroscopy; Fig. 2S) and TOCSY (total correlation spectroscopy), as well as heteronuclear HSQC, HSQC-TOCSY (heteronuclear single quantum coherence - total correlation spectroscopy) and HMBC (heteronuclear multiple bound correlation)] allowed for the assignment of chemical shifts to all protons and carbon atoms (Table 1). The detailed analysis of chemical shifts of carbon atoms also enabled determination of the size of the sugar rings. The absence of carbon signals in the region of δ 83–88 (except for the glycosylated C-3 of the residue **E** at δ 83.52) indicated the pyranose rings for all the sugar residues. Based on the results of the sugar analysis and NMR data the **A** and **D** spin systems were found to have a *manno* configuration, while the **B** and **E** spin systems had a *gluco* configuration. The remaining spin system **C** possessed the *galacto* configuration and was identified as a 3,6-dideoxygalactose (3,6-dideoxy-*xylo*-hexose residue). This sugar residue was not identified in the sugar analysis because it was degraded under the classical analysis conditions.

The anomeric configuration of the individual sugar residues was determined by chemical shifts and coupling constants. The ^{1}H NMR spectrum of the anomeric region showed three signals with $\delta > 5$ and two signals with $\delta < 5$. These values indicate the α anomeric configuration of the residues **A**, **B** and **C** and the β anomeric configuration for residues **D** and **E**. This assumption was clearly confirmed by the corresponding values of the coupling constants obtained from HSQC NMR spectrum (recorded without decoupling) as well as ^{1}H NMR spectrum: **A** ($^1J_{(H\text{-}1,C\text{-}1)}$ = 174 Hz), **B** ($^1J_{(H\text{-}1,C\text{-}1)}$ = 173 Hz, $^3J_{(H\text{-}1,H\text{-}2)}$ = 3.3 Hz), **C** ($^1J_{(H\text{-}1,C\text{-}1)}$ = 171 Hz, $^3J_{(H\text{-}1,H\text{-}2)}$ = 3.4 Hz), **D** ($^1J_{(H\text{-}1,C\text{-}1)}$ = 160 Hz), and **E** ($^1J_{(H\text{-}1,C\text{-}1)}$ = 161 Hz, $^3J_{(H\text{-}1,H\text{-}2)}$ = 7.8 Hz). Low-field shifted signals of the carbon atoms (underlined in Table 1) **A**2 (δ 77.93), **A**4 (δ 77.38), **B**3 (δ 81.64), **D**3 (δ 82.05), and **E**3 (δ 83.52) compared with those of the unsubstituted sugars [7] proved glycosylation at C-2 and C-4 of residue **A**, at C-3 of residue **B**, at C-3 of residue **D**, and at C-3 of residue **E**. The remaining residue **C** was identified as a terminal monosaccharide.

The sequence of the monosaccharides in the OPS was assigned using ROESY (rotating frame Overhauser enhancement spectroscopy) and HMBC NMR spectra. The following strong *inter*-residual NOE correlations were observed in the section of the ROESY spectrum (Fig. 2): **A1/B3**, **B1/E3**, **C1/D3**, **D1/A4** and **E1/A2**. Moreover, the section of the HMBC spectrum (Fig. 3) allowed for identification of the *inter*-residual carbon-proton correlations: C-1 of **A** and H-3 of **B** (δ 100.28/3.889), C-1 of **B** and H-3 of **E** (δ 100.44/3.644), C-1 of **C** and H-3 of **D** (δ 101.69/3.740), C-1 of **D** and H-4 of **A** (δ 101.13/3.980), as well as C-1 of **E** and H-2 of **A** (δ 102.76/4.293).

The absolute configuration of the 3,6-dideoxy-*xylo*-hexose residue was deduced by a comparison of the observed α-glycosylation effects (δ) on the substituted carbon atom C-3 and the neighboring carbon atom C-2 of Man residue with the expected values [7,8]. The observed effect δ +7.7 for C-3 (expected δ +7.5 for DD and δ +5.5 for DL) and δ −0.5 for C-2 (expected δ −0.2 for DD and δ −3.3 for DL) clearly indicates that both

Table 1
^{1}H and ^{13}C NMR data of the OPS isolated from *Pectobacterium aquaticum* IFB5637. The substituted carbon atoms are underlined.

Residue	Coupling constants [Hz]	Chemical shifts ^{1}H and ^{13}C (ppm)					
	$^{1}J_{(H-1-C-1)}$ $^{3}J_{(H-1-H-2)}$	H1 C1	H2 C2	H3 C3	H4 C4	H5 C5	H6 C6
A	174	5.333	4.293	4.096	3.980	4.041	3.831/3.831
→2,4)-α-D-Man*p*-(1→	nd	100.28	77.93	69.46	77.38	72.50	61.16
B	173	5.319	3.624	3.889	3.594	4.037	3.804/3.851
→3)-α-D-Glc*p*-(1→	3.3	100.44	71.38	81.64	70.92	72.68	61.32
C	171	5.097	4.017	2.054/1.997	3.875	4.140	1.165
α-Abe*p*-(1→	3.4	101.69	64.71	34.11	69.52	68.07	16.52
D	160	4.797	4.147	3.740	3.756	3.472	3.955/3.754
→3)-β-D-Man*p*-(1→	nd	101.13	71.96	82.05	66.99	77.34	62.17
D*	160	4.753	4.064	3.660[a]	3.652[a]	3.416	3.955/3.754
β-D-Man*p*-(1→	nd	101.30	71.85	73.26	68.19	77.28	62.17
E	161	4.493	3.451	3.644	3.652	3.447	3.906/3.733
→3)-β-D-Glc*p*-(1→	7.8	102.76	72.68	83.52	71.15	76.86	61.69

[a] Approximate ^{1}H chemical shifts.

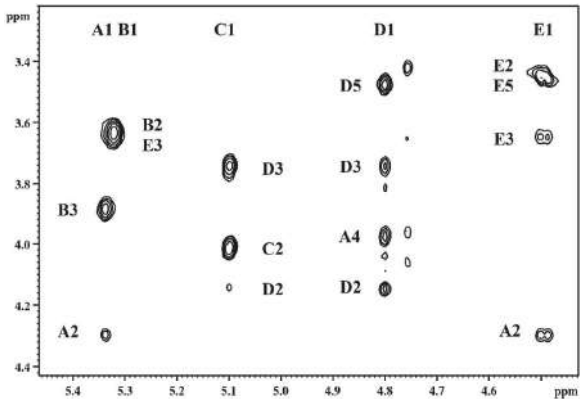


Fig. 2. The section of ROESY spectrum of the OPS isolated from *Pectobacterium aquaticum* IFB5637. The letters refer to the monosaccharides residues as defined in Table 1, and the Arabic numerals refer to the protons in the respective residues.

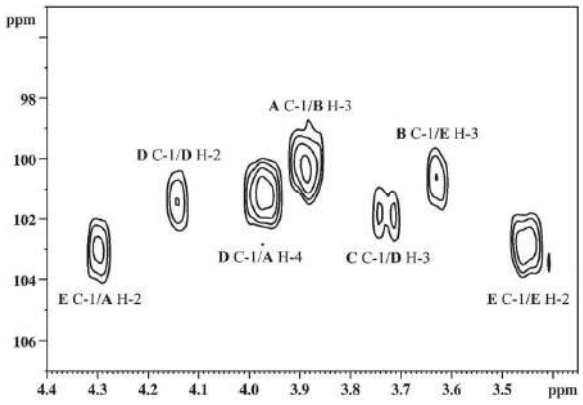


Fig. 3. The section of HMBC spectrum of the OPS isolated from *Pectobacterium aquaticum* IFB5637. The letters refer to the monosaccharides residues as defined in Table 1, and the Arabic numerals refer to the carbon atoms or the protons in the respective residues.

sugar residues in the disaccharide α-3,6-dideoxy-*xylo*-hexopyranose-(1 → 3)-β-Man*p* form a pair with the same absolute configuration (DD). This data revealed the presence of 3,6-dideoxy-D-*xylo*-hexose (abequose, Abe) in the studied OPS.

Moreover, the origin of a small signal labeled as **D*** (δ 4.753) in the anomeric region of ^{1}H NMR spectrum was analyzed in detail. The **D*** spin system was found to be a terminal mannose residue. The integration of all signals in the anomeric region revealed that the molar ratio of **A** + **B** (not resolved signals):**C**:**D**:**D***:**E** is respectively 2:0.8:0.8:0.2:1. The reduced intensity of the signals **C** and **D** (~0.8 each) compared to **A**, **B** and **E** (~1.0 each) and the presence of a terminal mannose (~0.2) indicates that the residue **D** is non-stoichiometric substituted by Abe (~80%). Probably part of Abe was decomposed during isolation of the LPS and preparation of the OPS.

The results of the chemical analyses and NMR spectroscopy revealed that the OPS isolated from *P aquaticum* IFB5637 consisted of the pentasaccharidic repeating units:

α-Abe*p* **C**

↓ 3

β-D-Man*p* **D**

↓ 4

→3)-β-D-Glc*p*-(1→2)-α-D-Man*p*-(1→3)-α-D-Glc*p*-(1→

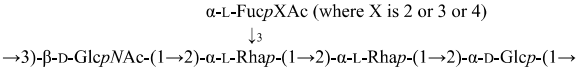
E **A** **B**

In conclusion, the structure of the chemical repeating unit of the OPS of the LPS from *P. aquaticum* IFB5637 was established, representing a novel structure not found before in any other OPS of the plant pathogenic bacteria studied so far. According to our best knowledge, this is the first assigned structure of the OPS isolated from *P. aquaticum*. The characterized OPS consists of two common hexoses (Man, Glc), as well as an abequose, first time identified among the *Pectobacteriaceae* family. Abequose is an unusual monosaccharide, which can be found almost exclusively in O-antigenic components of bacterial lipopolysaccharides [9]. Abequose has been found earlier in the O-antigens of different bacteria, including *Salmonella* serogroup B [10], *Citrobacter freundii* O35 and O38 [11], *Citrobacter freundii* O22 strain PCM 1555 [8], *Edwardsiella tarda* PCM 1153 [12], as well as in *Yersinia pseudotuberculosis* complex

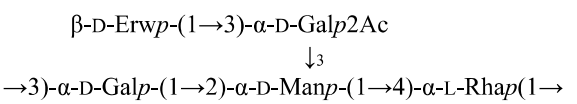
[13]. Based on the proposed structure and the other data published so far (structures below), it is possible to conclude that there is a high structural diversity in the OPS isolated from different *Pectobacterium* spp. strains.

The published structures of O-polysaccharides of *Pectobacteriaceae*:

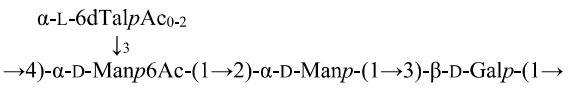
Pectobacterium atrosepticum SCRI1039 [14]:



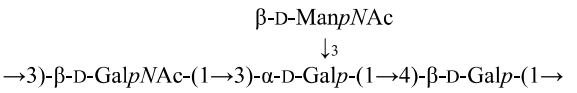
P. atrosepticum SCRI1043 [15]:



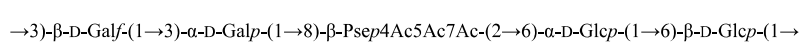
Pectobacterium brasiliense F152 [16]:



Pectobacterium parmentieri IFB5408 and IFB5427 [17]:



P. parmentieri SCC3193 and IFB5432 [18]:



Pse is 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-L-manno-non-2-ulosonic acid.

3. Materials and methods

3.1. Isolation and cultivation of P. aquaticum IFB5637

P. aquaticum IFB5637 strain was isolated in October 2016 from the Jeleń Lake, located in the region of Kashubia (54° 12′04.4 ″ N 17° 31′31.5 ″ E), Poland [3]. This isolate was grown on Crystal Violet Pectate (CVP) medium and identified to the species level with the use of polymerase chain reactions (PCR) and sequencing of *recA* and *dnaX* housekeeping genes as described previously [3].

To obtain bacterial biomass for LPS isolation, *P. aquaticum* IFB5637 kept in a stock at −80 °C was plated on trypticase soy agar (TSA, Oxoid, Basingstoke, UK) medium. After 24 h incubation at 28 °C, 200 mL of trypticase soy broth (TSB, Oxoid, Basingstoke, UK) was inoculated with a single bacterial colony picked from the TSA plate and incubated at 28 °C with 130 rpm shaking for 24 h. This overnight culture was subsequently poured into 2000 mL of TSB medium. Incubation for 48 h at 28 °C with 130 rpm shaking followed. Then, bacterial cells were harvested using centrifugation at 16,915.34×*g* for 10 min with a 6-16 KS (Sigma, Kanagawa, Japan) device. The collected bacterial cells were stored afterwards at −20 °C for further analyses. The cultivation procedure was repeated until a sufficient amount of bacterial biomass of *P. aquaticum* IFB5637 was obtained.

3.2. Extraction and purification of the LPS

Two types of extraction were used to isolate the LPS: PCP and hot phenol-water extraction. The bacterial cells were extracted by stirring with 100 mL of PCP mixture (phenol, chloroform, and petroleum ether; v/v/v 2:5:8) for 90 min and centrifuged (7012×*g*, 20 min, 4 °C). Then, the bacterial cells were extracted two more times with 90 mL of PCP mixture for 30 min and centrifuged under the same conditions. Petroleum ether and chloroform were removed from the supernatant using a rotary evaporator (30 min, 40 °C). Deionized water (~300 μL), which was added to the obtained phenolic layer did not cause any precipitation. Therefore, it was assumed that the studied bacterial strain does not produce the rough LPS. The smooth LPS (355 mg) was isolated from dry bacterial cells (18.39 g) of *P. aquaticum* IFB5637 using hot phenol-water extraction. The bacterial cells were suspended in warm water (100 mL) and phenol (20 mL). The mixture was stirred for 90 min at 65 °C and centrifuged (7012×*g*, 20 min, 4 °C). The water phase was collected, then the step was repeated with 40 mL of warm water. The sample obtained after two extractions (~100 mL) was dialyzed for 2 days, freeze-dried, and subjected to enzymatic digestion to remove proteins and nucleic acids. For this purpose, 895 mg from 1334 mg of the lyophilized sample was dissolved in 50 mL of buffer (100 mM Tris, 50 mM NaCl, 10 mM $MgCl_2$, at pH 7.5 with 1 M HCl) and addition of the pinches of DNAse I and RNAse (6.5 h, 37 °C). Then, some Proteinase K was added, and the sample was incubated at 55 °C overnight, dialyzed (2 days) and freeze-dried.

3.3. Isolation and preparation of the OPS

The LPS was hydrolyzed with 1% acetic acid (1 mL of acid for every 10 mg of sample) at 100 °C for 1.5 h and the obtained precipitate of lipid A was separated by centrifugation (7012×*g*, 20 min, 4 °C). The supernatant was evaporated with methanol to remove acetic acid, dissolved in 10 mL of deionized water and freeze-dried (272.5 mg). Part of the obtained fraction (44.5 mg) was dissolved in 1.5 mL of deionized water and fractionated by gel-permeation chromatography (GPC) on a column (120 × 1 cm) of Bio-Gel P-30 with deionized water as an eluent using a differential refractometer detector Jasco RI-2031A for monitoring the separation process. The collected fractions were freeze-dried and the ^{1}H NMR spectrum was recorded. The yield of the isolated high-molecular-mass O-polysaccharide was ~15 mg (~33% of the LPS weight).

3.4. NMR spectroscopy

The sample of the OPS (3 mg) was exchanged twice with 1 mL of 99.5% D_2O, freeze-dried and finally dissolved in 0.6 mL of 99.95% D_2O. 1D and 2D NMR spectra (^{1}H–^{1}H COSY, TOCSY, ROESY and ^{1}H–^{13}C HSQC, HSQC-TOCSY, HMBC) were recorded at 40 °C using a Bruker Avance III 500 MHz spectrometer. Bruker TopSpin 3.6.2 software was used to acquire and process the NMR data. The chemical shifts were referenced to the internal standard of acetone (δ_H 2.225; δ_C 31.45). The COSY, the TOCSY, and the ROESY experiments were recorded by using

data sets of 4096 × 256 points. The $^{1}H,^{13}C$ HSQC, the $^{1}H,^{13}C$ HSQC-TOCSY, and the $^{1}H,^{13}C$ HMBC experiments were recorded in ^{1}H-detection mode using data sets of 2048 × 256 points. The TOCSY experiment was performed with spinlock times of 100 ms.

3.5. Chemical analyzes

The sugar analysis was carried out to identify and determine the number of sugar residues in the repeating unit of the OPS. The OPS (0.8 mg) was hydrolyzed with 2 M trifluoroacetic acid (2 h, at 120 °C), the obtained monosaccharides were reduced with sodium borohydride overnight and the resulting alditols were acetylated with 200 μL of acetic anhydride in the presence of sodium acetate (2 h at 120 °C). The methylation analysis was performed to determine the substitution positions of sugar residues [19]. The OPS (1 mg) was methylated using DMSO (300 μL) as a solvent in the presence of KOH and methyl iodide (3 × 50 μL). The per-*O*-methylated polysaccharide was hydrolyzed, reduced, and acetylated as described above in the sugar analysis procedure.

Determination of the absolute configuration of the monosaccharides was carried out using GLC analysis of the obtained 2-butyl glycosides. The OPS was hydrolyzed. The obtained monosaccharides were reacted with (*S*)-(+)-butan-2-ol and then acetylated [20].

The derivatives of alditol acetates, partially methylated alditol acetates, and 2-butyl glycosides were analyzed using GLC and GLC-MS.

3.6. GLC and GLC-MS techniques

All GLC analyzes were performed using gas chromatograph GC-2010 Pro (Shimadzu) with a flame ionization detector (FID) detector equipped with a Rtx-2330 capillary column (length: 60 m, inner diameter: 0.25 mm, film thickness: 0.2 μm) for but-2-yl glycosides and with a capillary Rtx-5 column (length: 30 m, inner diameter: 0.25 mm, film thickness: 0.25 μm) for alditol acetates. In GLC analyzes the carrier gas was Ar. The temperature program from 120 °C to 260 °C, rate 2 °C min^{-1}, then 30 min isotherm at 260 °C was used. GLC-MS analyzes were performed on gas chromatograph coupled with mass spectrometry GC MS-QP2010 SE (Shimadzu) with a capillary column Rtx-5. In GLC-MS analyzes the carrier gas was He. The temperature program from 120 °C to 265 °C, rate 4 °C min^{-1}, 10 min isotherm at 265 °C was used. The mass spectra were recorded under the following conditions: EI ionization (70 eV), ion source temperature 220 °C and recorded mass range from 43 to 550 *m/z*.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All the data are included in this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carres.2022.108696.

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Supplementary materials

The structure of an abequose - containing O-polysaccharide isolated from *Pectobacterium aquaticum* IFB5637

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Highlights

The O-polysaccharide was isolated from *Pectobacterium aquaticum* IFB5637

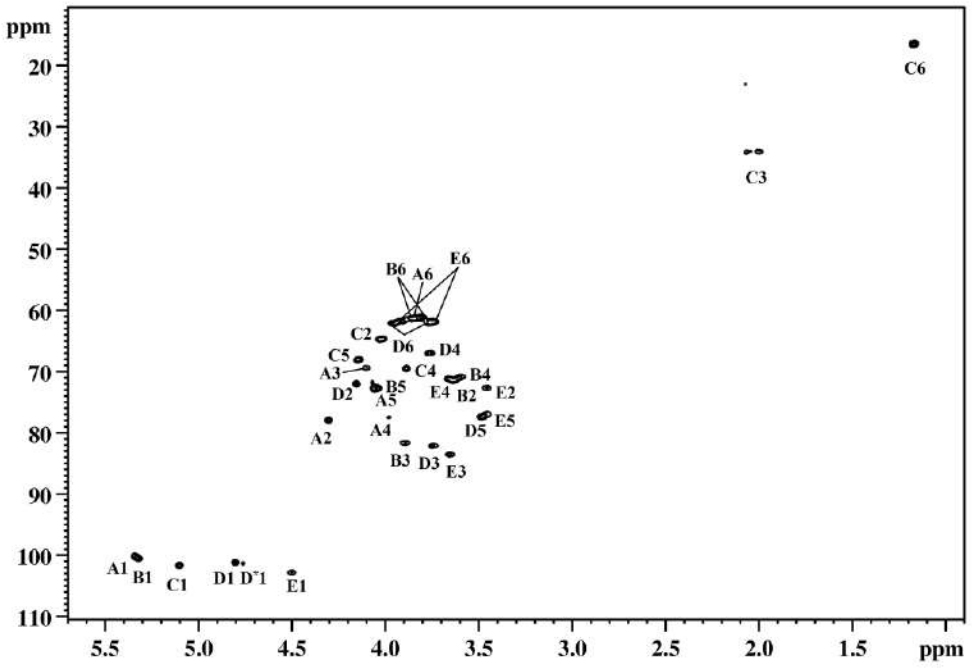
The chemical structure was determined using chemical methods and NMR spectroscopy

The OPS consists of mannose, glucose as well as an abequose - an unusual component of bacterial LPS

Figure legends:

Fig. 1S. The HSQC spectrum of the OPS isolated from *Pectobacterium aquaticum* IFB5637. The letters refer to the monosaccharides residues as defined in **Table 1**, and the Arabic numerals refer to the proton/carbon cross-peaks in the respective residues.

Fig. 2S. The COSY spectrum of the OPS isolated from *Pectobacterium aquaticum* IFB5637. The letters refer to the monosaccharides residues as defined in **Table 1**, and the Arabic numerals refer to the protons in the respective residues.



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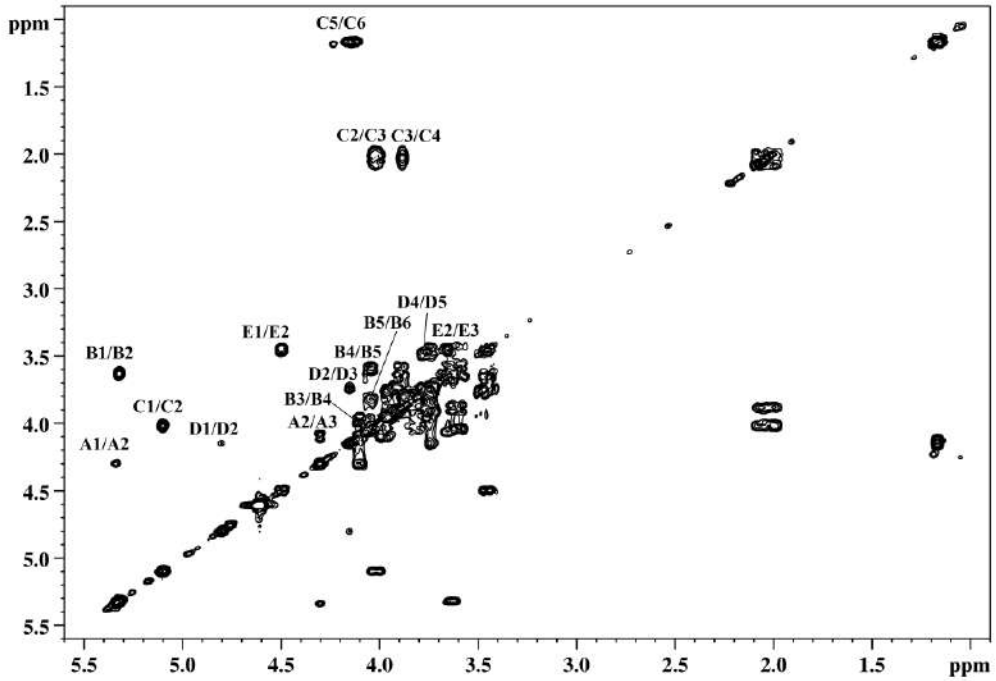

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Gdańsk, 19.09.2023

AUTHOR CONTRIBUTION STATEMENT

I declare that in the manuscript:

"The structure of an abequose - containing O-polysaccharide isolated from *Pectobacterium aquaticum* IFB5637"
Agnieszka Kowalczyk, Nikola Szpakowska, **Weronika Babinska**, Agata Motyka-Pomagruk, Wojciech Sledz, Ewa Lojkowska, Zbigniew Kaczyński

my contribution involved in isolation and purification of LPS and OPS. Using chemical methods and 1D and 2D NMR spectroscopy I determined the chemical structure of OPS. Finally I took part in the discussion on the collected data. I prepared the first version of this manuscript regarding chemical analysis and corrected it according to the co-authors' suggestions.

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my contribution involved conceived and designed biological experiments. Moreover I took part in preparation of bacterial biomass for the analysis of the LPS structure. I prepared first version of this manuscript describing the biological part of the performed research.

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my involvement included participation in culturing bacteria, corrections of the the manuscript and discussion of the research results.

Agata Motyka-Pomagruk

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my contribution involved in preparation of bacterial biomass for the analysis of the LPS structure.

University of Gdańsk | MEDICAL UNIVERSITY OF GDAŃSK

Gdańsk, 16/10/2023

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Relates to publication:

Kowalczyk Agnieszka, Szpakowska Nikola, **Babińska Weronika,** Motyka-Pomagruk Agata, Śledź Wojciech, Łojkowska Ewa, Kaczyński Zbigniew. The structure of an abequose - containing O-polysaccharide isolated from *Pectobacterium aquaticum* IFB5637. ***Carbohydrate Research*** **2022**, 522: 108696 (doi: 10.1016/j.carres.2022.108696)

I declare that, I was responsible for co-conceptualization of the biological part of the project. I participated in the discussion of the final version of the manuscript.

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my contribution consisted of supervising the structural studies of the OPS. In addition, I participated in the discussion of the collected data and contributed to the preparation of the manuscript.

Z. Kac

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Manuscript No. 1

The search for foundations of virulence in an economically significant plant pathogen *Dickeya solani via* transcriptomic profiling.

Weronika Babińska-Wensierska, Agata Motyka-Pomagruk, Alessio Mengoni, George C. diCenzo, Ewa Łojkowska

submitted for publication

The search for foundations of virulence in an economically significant plant pathogen *Dickeya solani via* transcriptomic profiling

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Keywords: *Dickeya* spp., soft rot, blackleg, methylomes, RNA-Seq

1. ABSTRACT

Bacteria from the genus *Dickeya* cause soft rot on a wide range of crops or ornamentals and blackleg of potatoes. Among the members of this genus, *D. solani* shows superior pathogenic potential associated with efficient production of plant cell wall degrading enzymes, most of all pectate lyases, in addition to more potent colonization of plant roots and faster movement through the host vascular system. Despite that, *D. solani* strains exhibit remarkable genetic homogeneity, in contrast to differences in the virulence-related phenotype. On this basis it might be presumed that diverse pathogenic potential of *D. solani* strains may be related with variations in expression of genes encoding key virulence factors.

To confirm this hypothesis, this study investigated the metylomes and transcriptomes of two *D. solani* strains (virulent IFB0099 and low virulent IFB0223), that differed significantly in plant tissue maceration potencies on potato and chicory in addition to efficacy of virulence factors production. Analysis of Pacific Biosciences DNA sequencing data revealed no discernible distinctions in DNA methylation patterns that could account for the significant variation in virulence observed between the analyzed strains. Transcriptomic analysis of RNA-Seq data collected for *D. solani* IFB0099 and IFB0223, cultured in a minimal medium with addition or not of polygalacturonic acid, which mimics different stages of the infection process, revealed that stimulation of expression of some genes encoding pectate lyses (*pelD*, *pelE*, *pelL*) was higher in IFB0099 compared to IFB0223 under the induced conditions. In addition, we observed in the presence of PGA an increased induction of expression of some genes encoding the constituents of type II secretion system (T2SS) in strain IFB0099 contrarily to IFB0223, which may potentially result in the elevated virulence of the IFB0099 strain towards potato plant tissue. Additionally, there was an increased stimulation of expression of the

flagella-related genes in the IFB0099 strain compared to IFB0223, which could lead to an enhanced motility and pathogenic potential of this *D. solani* strain.

This is the first study investigating transcriptomes of two *D. solani* strains differing in virulence that pointed candidate genes, which should be further investigated with the molecular biology tools for their contribution into pathogenic phenotypes of the soft rot *Pectobacteriaceae*.

2. INTRODUCTION

Bacteria classified to the genus *Dickeya* are responsible for economically significant plant diseases such as soft rot, which may be observed on many crops, vegetables or ornamentals, and blackleg of potatoes [1]. Due to a notable impact on the yield and quality of agricultural products, *Dickeya* spp. were included in 2012 in the top ten list of most important bacterial plant pathogens [2]. The species *Dickeya solani*, in particular, was a major causative agent of blackleg in Europe already in 2010 [3]. In addition, the shipment of seed potatoes infected with *D. solani* from Europe to Israel led to severe disease outbreaks. Among five examined in Israel potato cultivars disease incidences ranged from 5% to 30% and symptomatic potato plants were detected on over 200 ha of arable land [4].

Prior to the delineation of the genus *Dickeya* in 2005 [5], its members were classified as either *Pectobacterium chrysanthemi* or *Erwinia chrysanthemi* [6, 7]. Following continuous progress in genetic-based identification methods, 12 *Dickeya* species have been validly published to date. In 2014, *D. solani* was established as a new species within the *Dickeya* genus [3]. Despite being isolated from different environmental sources, including potato, ornamentals, and the rhizosphere, the majority of *D. solani* isolates from Europe indicated genetic homogeneity and differ solely by several dozen SNPs/InDels [8, 9, 10, 11, 12].

The competitive advantage of *D. solani* strains is associated with higher aggressiveness of these strains in contrast to other *Dickeya* spp. This pathogen requires lower optimal temperatures for disease development, and is able to set up infection from lower inoculum levels [13, 14]. Furthermore, *D. solani* strains seem to have a greater ability to colonize the roots of potato plants and spread through the vascular system of the host [15]. The pathogenic potential of *Dickeya* spp. is linked with efficient production and secretion of numerous virulence factors, most of all plant cell wall degrading enzymes (PCWDEs) [16, 17, 18, 19]. Nevertheless, successful colonization of the host also demands the involvement of additional elements facilitating bacterial movement or adhesion, *i.e.* flagella, lipopolysaccharides, and exopolysaccharides [17, 20]. Subsequently, a key role is attributed to intermediate factors allowing for bacterial adaptation to conditions encountered within the host tissue, including oxidative stress, iron deficiency, and the presence of toxic compounds [21].

In general, soft rot disease symptoms result from the action of PCWDEs, particularly pectate lyases (Pels), as these enzymes break down pectins that are the main structural component of the plant cell wall. In the *Dickeya* genus, genes responsible for catabolism of oligogalacturonides are regulated

by the KdgR repressor, which becomes inactive during production of 2-keto-3-deoxygluconate (KDG), an intermediate product of pectin degradation [22, 23]. The expression of genes encoding major pectate lyases PelD and PelE is primarily induced by polygalacturonic acid (PGA), one of the three components of pectins, along with rhamnose and various side chains [24]. In addition, transcription of the gene coding for one secondary pectate lyase PelL, is induced by both the presence of PGA and the plant extract [25]. Therefore, supplementation of media with PGA, a structural element of plant cell wall, mimics the progression of the infection process and is commonly applied for induction of expression of genes encoding pectate lyases under laboratory conditions [25, 26, 27]. Moreover, the production of pectinolytic enzymes among Soft Rot *Pectobacteriaceae* (SRP) strongly depends on the bacterial growth phase. Therefore, various research groups examined activities of PCWDEs during either lag, exponential, stationary or decline phase of bacterial growth. For instance, in the research of Pirhonen (1991) [28] for pectinases, proteases, and cellulase assays, the analyzed strains of *Erwinia carotovorum* subsp. *carotovorum* were grown to a stationary phase. On the other hand, research conducted by Potrykus et al. (2020) [29] on two strains of *D. solani* (IFB0099 and IFB0223) differing in virulence, revealed that the pectate lyase activity measured in macerated potato tissue was maximal at 48 h post-infection, with a decline at 72 h. Finally, the study presented by Jafra et al. (1999) [30] indicated that after 20 h of infection of potato tubers with *D. chrysanthemi* (formerly *Erwinia chrysanthemi*), significant differences in the induction of Pels synthesis can be observed. Among the tested pectinase genes, the expression level of *pelE* and *pelI* after 20 h of infection is the highest, while the expression of *peL* and *pelZ* is moderate, but higher than *pelA* [30]. Moreover, research presented by Kepseu et al. (2010) [31] reveals that pectate lyases are not activated in the initial exponential growth phase, leading to a lack of induction of the genes responsible for pectin degradation during this stage of growth.

The virulence of plant pathogenic bacteria is also affected by DNA methylation, which may control the temporal expression of specific subgroups of genes in response to environmental stimuli [32]. DNA is methylated at specific positions on cytosine and adenine nucleotides, namely at C-5 or N-4 sites of cytosine and the N-6 site on adenine. This process is carried out by enzymes called DNA methyltransferases (MTases) including cytosine MTase (*e.g.*, Dcm of *Gammaproteobacteria*), adenine methylase (*e.g.*, Dam in *Gammaproteobacteria*) and cell cycle-regulated methylase (*e.g.*, CcrM in *Alphaproteobacteria*) [33]. Research findings demonstrated that the pathogenicity of Dam-deficient *Dickeya chrysanthemi* (formerly *Erwinia chrysanthemi*) is significantly diminished as demonstrated on the host plants African violet and lettuce [34].

D. solani strains, including those of diverse geographical origin and isolation year, exhibit high genetic homogeneity despite significant differences observed in their virulence [27, 35, 36]. High uniformity of this taxon is also evident in REP PCR- (Repetitive sequence-based PCR) or PFGE-based genomic profiling (Pulsed-field Gel Electrophoresis), and the low variation was also noted in the sequences of housekeeping genes as shown by Multilocus Sequence Typing (MLST) [3, 35, 37, 38,

39]. Likewise, the analysis of variable number of tandem repeats (VNTR) disclosed only 3 groups among the studied 54 *D. solani* isolates [27]. Similarly, a comparative genomics evaluation on 22 *D. solani* strains (including both high and low virulent isolates) revealed only minor differences in the genomic structures, pangenome fractions, ANIb, ANIm, Tetra values, and the core genome-based phylogeny [12] in spite of significant deviations noted between these strains in terms of virulence factors production (pectinases, cellulases and proteases) and motility [11, 27].

Considering that many of the strain-specific genes identified in a *D. solani* pangenome [12] encode transcriptional regulators (*e.g.* AcrR, ArsR, LysR, MarR, RpiR, AlpA, DksA), and the fact that studying *D. solani* genomes have not provided yet conclusive justification for the observed variations in virulence, we attempted to explain variation in pathogenic potential of *D. solani* strains by the means of methylation analysis and transcriptomic profiling. We included two strains of *D. solani*, IFB0099 and IFB0223, that differ significantly in their level of virulence on potato and chicory in addition to production of virulence factors [11, 27]. At first, the already published Pacific Biosciences data for *D. solani* IFB0099 and IFB0223 [11] were analyzed to identify methylated sites and motifs in their genomes. Then, *D. solani* IFB0099 and IFB0223 were cultured in a minimal medium supplemented or not with PGA and their transcriptomes have been subjected to RNA-sequencing followed by bioinformatic analysis of this data. To the best of our knowledge, this is the first study providing insight into the methylomes and transcriptomes of high and low virulent *D. solani* strains aiming to identify factors that may explain their various pathogenic potential towards economically significant crops.

3. MATERIALS AND METHODS

3.1 Bacterial cultures and isolation of RNA

Two strains of *D. solani*, differing notably in virulence were selected for the current research. *D. solani* IFB0099 was isolated in Poland in 2005 [37] from a rotten potato plant. This strain causes intense maceration of potato tubers and chicory leaves [11, 27]. *D. solani* IFB0223 was isolated in 2005 from the rhizosphere of a healthy potato plant in Germany, and exhibits low maceration potency on potato tubers and chicory leaves [11, 27]. The genomic sequences of *D. solani* IFB0099 and IFB0223 have been deposited during our former study in the GenBank database under the accession numbers CPO24711 and CPO24710, respectively [11]. *D. solani* IFB0099 and IFB0223 are available in the bacterial collection of Intercollegiate Faculty of Biotechnology University of Gdansk and Medical University of Gdansk (IFB UG & MUG) in Poland, in which they are stored at -80°C in 40% (v/v) glycerol.

D. solani strains (either IFB0099 or IFB0223) were collected from the frozen stocks, streaked on Tryptone Soya Agar medium (TSA) (Oxoid, Basingstoke, UK) and incubated at 28°C for 24 h. Subsequently, a single bacterial colony was picked from the TSA plate and used for inoculation of

liquid M9 minimal medium (with glucose) [40]. These cultures were then incubated for 24 h at 28°C with shaking at 130 rpm. Subsequently, 100 µl of the overnight bacterial cultures were transferred to either M9 (non-induced conditions) or M9 supplemented with 4 g L^{-1} PGA (induced conditions), and incubated for 48 h at 28°C with 130 rpm shaking to mimic environmental conditions encountered at different stages of the infection process [25, 26, 27]. The overnight bacterial cultures were then centrifuged (6000 rpm for 10 min), and the resultant pellets were washed twice with 0.85% NaCl prior to suspending the cells by vigorous mixing in sterile physiological saline.

From *D. solani* IFB0099 and IFB0223 cells, suspended in 0.85% NaCl post culturing in either M9 or M9 + PGA, total RNA was extracted using a commercially available Bead-Beat Total RNA Mini kit (A&A Biotechnology, Lodz, Poland) according to the manufacturer's instructions. The isolated RNA was purified and concentrated using Clean-Up RNA Concentrator and Total RNA Mini Concentrator (A&A Biotechnology, Lodz, Poland) kits following the protocols provided by the producer.

The purity and concentration of RNA in each sample was evaluated spectrophotometrically using NanoDrop ND-1000 (Thermo Fisher Scientific, Minneapolis, USA). In addition, Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA) was implemented to examine the concentration, integrity (RNA integrity number; RIN), and 23S/16S rRNA ratio in the RNA samples isolated from *D. solani* strains.

3.2 Detection of the methylated DNA motifs

During our former study, the genomes of *D. solani* IFB0099 and IFB0223 were sequenced on a Pacific Biosciences platform by BaseClear (The Netherlands) [11]. 118,344 and 102,248 PacBio reads were acquired for *D. solani* IFB0099 and IFB0223, respectively. Initially, bax.h5 files were converted to bam files using bax2bam version 0.0.9 (github.com/EichlerLab/bax2bam) on the Galaxy webserver [41]. Then, identification of the methylated motifs was performed using the smrtlink version 7.0.1.66975 command line tools (Pacific Biosciences). Specifically, the bam files were first mapped to the corresponding reference genome (GCF_000831935.2 for *D. solani* IFB0099 and GCF_003718335.1 for *D. solani* IFB0223) using pbalign version 0.4.1. The methylated sites and motifs were then detected in each genome using ipdSummary version 2.4 and motifMaker.

3.3 Analysis and visualization of the RNA-Seq data

Samples of the isolated *D. solani* RNA (minimum 5 µg of RNA; concentration of 65 ng/µl) were send to Genomed (Warsaw, Poland) for sequencing service. There, rRNA was depleted using QIAseq FastSelect -5S/16S/23S kit (Qiagen, Hilden, Germany). Afterwards, a strand-specific cDNA library was prepared using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England BioLabs Inc., Massachusetts, USA). The sequencing process was performed on a MiSeq genomic sequencer (Illumina, San Diego, USA) to reach a coverage of 10 million paired-end reads per sample. The sequencing depth was selected on the basis of the recommended number of reads for

differential expression [42]. Three biological replicates were performed for each of the two strains across the two examined conditions.

Raw RNA-Seq reads were stripped of adapter sequences during conversion of the raw reads to the FASTQ format. To achieve 10 million pairs of reads per sample, 4 subsets of forward reads in addition to four subsets of reverse reads were generated for each biological sample. Trimming of the reads was performed using Trimmomatic (version 0.39) [43] with the following parameters: adapter removal based on the provided adapter sequence file, quality trimming using a sliding window approach with a window size of 5 and a minimum average quality of 20, and a minimum length of 50 bases after trimming. After this step, the quality of trimmed reads was checked with FASTQC [44]. Finally, the reads from each of the four subsets that passed quality control were combined into one dataset containing high quality reads per biological sample.

The trimmed RNA-Seq reads were mapped *ab initio* to the reference genome of *D. solani* type strain IPO 2222 (GCF_001644705.1) using Bowtie2 (version 11.2.0) [45]. The quantitative gene expression analysis involved identification of DEGs (differentially expressed genes) by RSEM (RNA-Seq by Expectation-Maximization) [46] using an absolute log2 Fold Change > |1| and *p* value < 0.05. DEGs were further studied with the DESeq2 package (*p* adj < 0.05) [47] in R (RStudio version 2021.09.0). The computed diagnostic tests included comparison identifier MA and a Volcano plot. I addition, a two-dimensional chart of similarities between the samples from the analyzed principal component analysis (PCA) collections was generated for all the expressed genes. To visualize gene expression levels for the analyzed strains, a heatmap of the normalized expression values on the re-filtered DEGs in R (RStudio version 2021.09.0, ggplot2 package) was generated. The filtered genes were transformed to log2 scale in a way that minimizes differences between samples for rows with small counts, and which normalizes with respect to library size ("regularized log" transformation). Finally, upregulated or downregulated genes under the induced (IFB0099 *vs.* IFB0223) and non-induced (IFB0099 *vs.* IFB0223) conditions were identified based on log2 fold change > |1| and *p* adj < 0.05. For the analysis of differential expression of genes encoding major virulence factors in case of IFB0099 (induced *vs.* non-induced conditions) and IFB0223 (induced *vs.* non-induced conditions) all DEGs were included, regardless of their statistical significance. Finally, functional annotations of the DEGs were corrected basing on high quality gene annotations from the genome of *D. dadantii* 3937, which is deposited in the NCBI database under the accession number ASM14705v1. The assignment of functions to the identified proteins was based on the UniProt database (Universal Protein Knowledgebase; accessed 09.2023).

4. RESULTS

4.1 The DNA methylomes of *D. solani* IFB0099 and *D. solani* IFB0223

Bacteria can rapidly adapt and respond to the surrounding environment in part *via* epigenetic regulation [32]. In particular, DNA methylation is known to affect the production of virulence factors,

and is therefore crucial in terms of the development of disease symptoms [48]. Considering this, a DNA methylation analysis was conducted to evaluate potential differences between *D. solani* strains IFB0099 and IFB0223.

Table 1. Methylated motifs identified in the genomes of *D. solani* IFB0099 and IFB0223

		***Dickeya solani* IFB0099**		***D. solani* IFB0223**	
Motif[a]	**Type[b]**	**Count[c]**	**Fraction[d]**	**Count[c]**	**Fraction[d]**
G**A**TC CT**A**G	m6A	22571	0.97	22,592	0.95
CNC**A**(N7)RTGG GNGT(N7)Y**A**CC	m6A	1053 1053	0.98 0.97	1060 1060	0.93 0.94
GC**A**(N5)GTTC CGT(N5)C**A**AG	m6A	440 440	0.96 0.96	441 441	0.95 0.90

[a]The methylated nucleotides are indicated in boldface font
[b]Marks whether the modification is N^6-methyladenoside (m6A)
[c]The total number of appearances of the motif in the genome, regardless of the methylation status
[d]The relative frequency of a particular methylated motif in the analyzed genome

In total three methylated motifs were identified in the genomes of *D. solani* IFB0099 and IFB0223, all of which were N6-methyladenosine (m6A) modifications (Table 1). Among these modifications, majority of the adenine methylations can be observed at the 5'-GATC-3' site methylated by the Dam MTase commonly identified in members of the class *Gammaproteobacteria*. The following sites were methylated in both strains: 5'-CNCA(N7)RTGG-3' and the complement 5'-CCAY(N7)TGNG-3' in addition to 5'-GCA(N5)GTTC-3' and the complement 5'-GAAC(N5)TGC-3'. The frequencies of all three motifs were similar, albeit not identical, between the two strains.

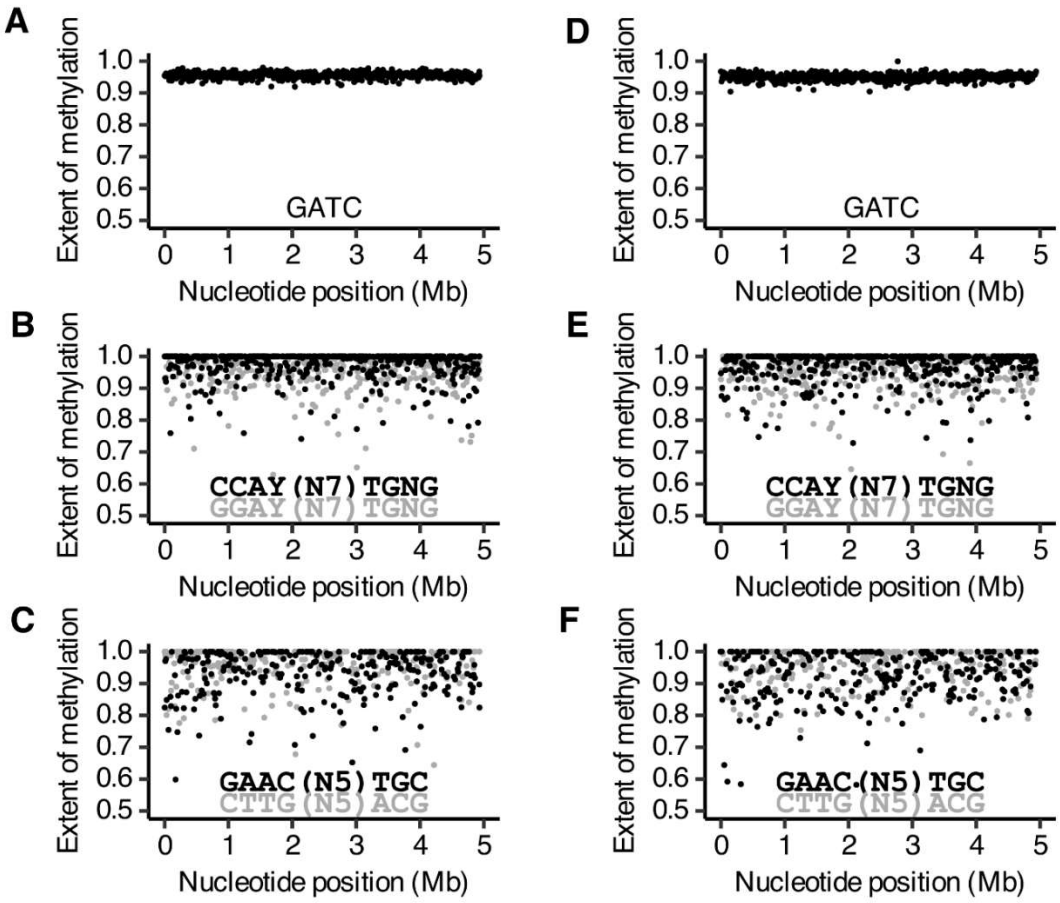

Figure 1. Genome-wide DNA methylome of IFB0099 and IFB0223 *D. solani* strains. The extent of methylation is shown, using a 10-kb sliding window, of the methylated sites across the *D. solani* IFB0099 (A to C) and IFB0223 (D to F) chromosomes. Data for each of the three methylated motifs is shown in separate panels, with the sequence of each motif provided within the panels. In the case of non-palindromic motifs where each strand is methylated, the data for the two strands are shown in black (top strand) and grey (bottom strand)

Figure 1 shows the extent of methylation among all the identified methylation motifs within a 10 kb window in the two analyzed *D. solani* strains (IFB0099 and IFB0223). The genome-wide methylation patterns for strain IFB0099 (Figure 1A-C) are comparable to those of IFB0223 (Figure 1D-F) strain. The 5'-GATC-3' motif shows robust methylation across the chromosome and population. Moreover, the level of 5'-GATC-3' methylation ranged between 90-95%, suggesting that there is always a small fraction of bacterial cells that lack methylation at a given site (Figure 1A and D). The other analyzed motifs show higher variation in the methylation rates. For most identified sites, the methylation is close to 100% (Figure 1B, C, E and F).

No significant differences in the overall DNA methylation patterns were detected between the two *D. solani* strains that could contribute to explaining the observed deviations in the virulence-associated phenotype of IFB0099 in contrast to IFB0223. In order to search for factors affecting the virulence of *D. solani* the transcriptomes of IFB0099 and IFB0223 strains growing in non-induced

(without the PGA in the medium) and induced (with the PGA in the medium) conditions were performed.

4.2 Transcriptomic profiling of *D. solani* IFB0099 and IFB0223

After quality trimming and adapter clipping, about 94% of the generated pairs of reads passed the quality checks and were subjected to further analysis (Table S1). On average, approximately 33 million reads were generated per library (Table S2).

Of the 31 883 758 – 35 397 968 paired-end reads per sample that passed the quality checks, more than 99% of the input was successfully mapped to the reference genome of *D. solani* IPO 2222, with over 66% aligning exactly once (Table S2). On the other hand, less than 0.59% of the paired-end reads did not align concordantly to the genome of *D. solani* IPO 2222 strain. Overall, these results indicated high quality of mapping of the reads, which ensures reliability of the subsequent DEGs analysis.

4.3 PGA induces diverse transcriptomic changes in *D. solani* IFB0099 and IFB0223

MA and Volcano plots are often used to provide an overview of gene expression changes between diverse conditions. No unusual patterns were detected in the MA or Volcano plots when comparing *D. solani* IFB0099 and IFB0223 in the induced *versus* non-induced conditions (Figure S1).

To investigate the overall effects of culture condition (induced *vs.* non-induced) and genotype (IFB0099 or IFB0223) on gene expression patterns in *D. solani*, PCA was performed using all the samples (Figure 2).

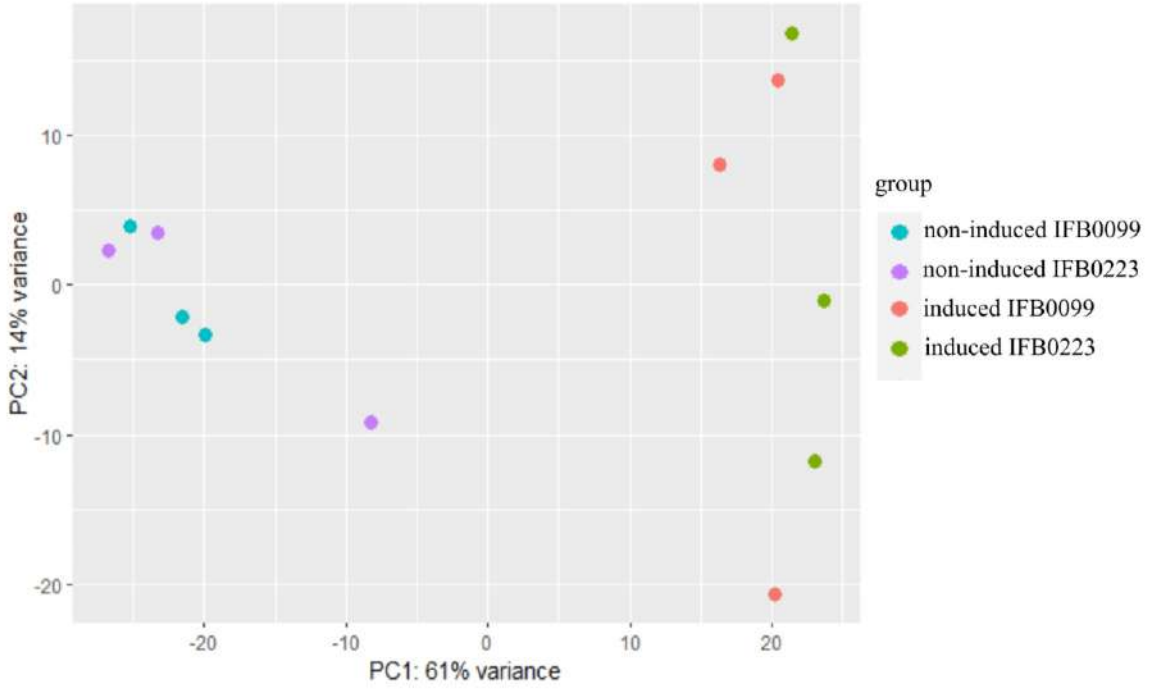

Figure 2. Principal coordinates analysis (PCA) showing the distribution of RNA-Seq trimmed reads generated for *D. solani* IFB0099 and IFB0223 cultivated either in non-induced or induced conditions

in a two-dimensional similarity using the Bray-Curtis distance matrix. PC1 and PC2 represent the two principal components

The PCA shows the key sources of variation between the tested samples, with Principal Component 1 explaining 61% of the variation in the data and Principal Component 2 representing 14% of the variation. It is evident from the clustering in the PCA plot that the gene expression profiles are strongly influenced by the conditions in which the strains were grown, while the genotype had a much smaller impact. Interestingly, there was tighter clustering of the samples from the non-induced conditions than the induced conditions along Principal Component 2, indicating that this source of variability was predominately observed in the presence of PGA.

4.4 Differentially expressed genes in *D. solani* IFB0099 and IFB0223

In *D. solani* IFB0099, 546 genes showing higher expression and 479 genes showing lowered expression were identified in the induced *versus* the non-induced conditions with the established threshold for statistical significance of differential expression (absolute log2 fold change > |1|, *p* adj < 0.05) (Table S3). Concerning the other *D. solani* strain analysed *i.e.* IFB0223, 466 and 436 genes were observed to be notably upregulated and downregulated, respectively, if the strain had been cultured under induced in contrast to the non-induced conditions (Table S4).

4.4.1 The most significant DEGs in *D. solani* IFB0099 and IFB0223 cultured either under induced or non-induced conditions

Heatmaps of normalized expression of 30 genes with the lowest adjusted *p*-value were computed to depict genes whose expression levels differed most significantly in *D. solani* IFB0099 (Figure 3) and IFB0223 (Figure 4) under the induced (with PGA) *versus* non-induced (without PGA) conditions. The clear clustering of the biological replicates from the induced and non-induced experimental conditions (Figures 3-4) confirms the reliability of the biological replicates of transcriptomes of both *D. solani* IFB0099 and IFB0223.

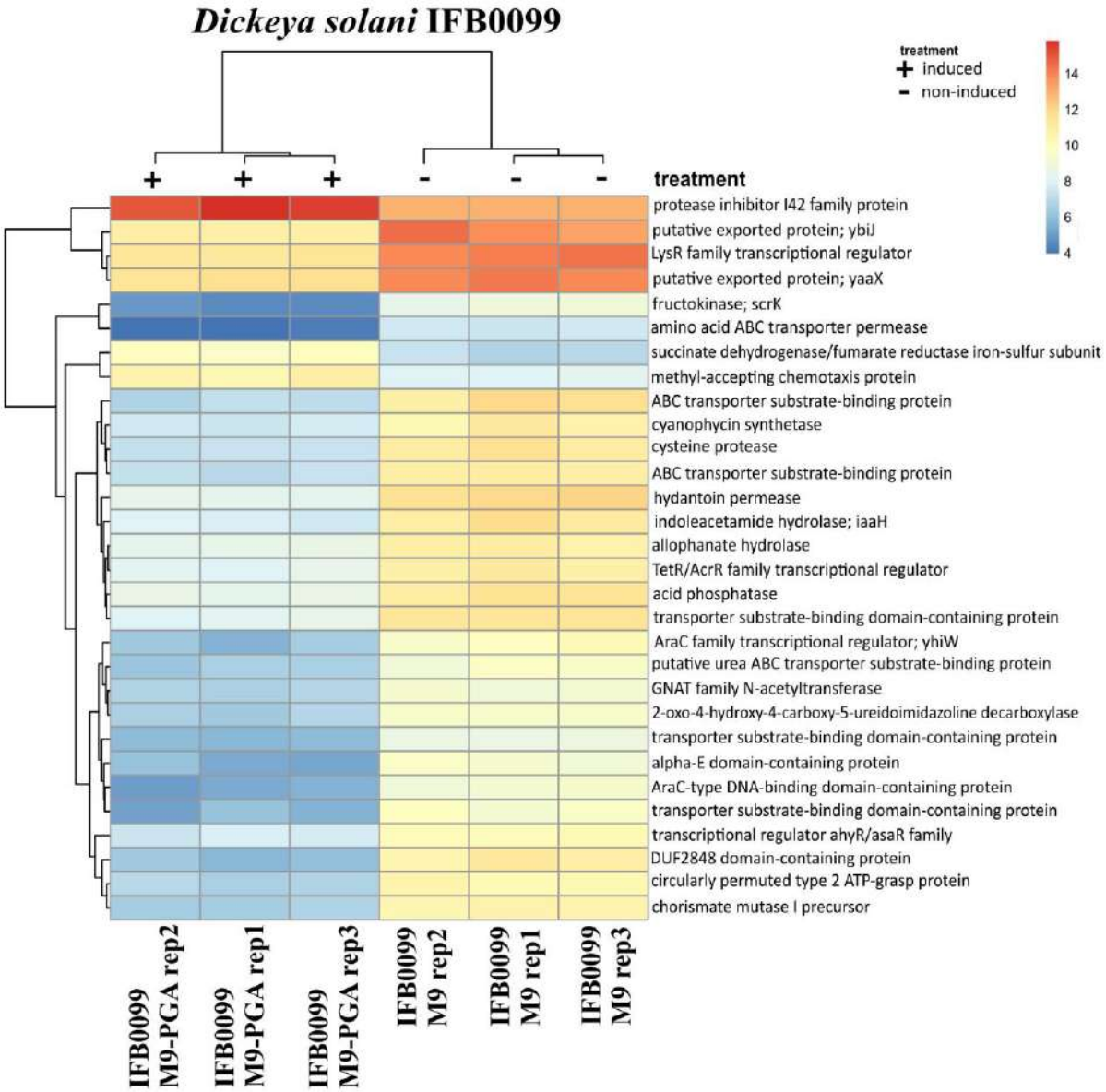

Figure 3. Comparison of the expression values of 30 genes with the lowest adjusted *p-value* for *D. solani* IFB0099 cultivated under induced *vs.* non-induced conditions. Hierarchical clustering was used to generate the heatmap. The scale shows the expmatrix variable, which reflects the expression level of genes transformed using the regularized log technique.

The most statistically significant difference in gene expression between the induced *vs.* non-induced conditions for *D. solani* IFB0099 involved a gene encoding a protease inhibitor I42 family protein (Figure 3). Among the revealed genes differing most significantly in their expression levels, there were two genes related to arabinose operon (AraC), *i.e.* the gene coding for AraC family transcriptional regulator and the gene encoding the AraC-type DNA-binding domain-containing protein, which both showed lower expression in the induced compared to non-induced conditions (Figure 3). Interestingly, seven of the other most differently expressed genes encoded components of the transporter systems (two ABC transporter substrate-binding proteins, three transporter substrate-binding domain-containing proteins, putative urea ABC transporter substrate-binding protein and an

amino acid ABC transporter permease), which all showed reduced expression in the induced conditions if juxtaposed to the non-induced ones (Figure 3).

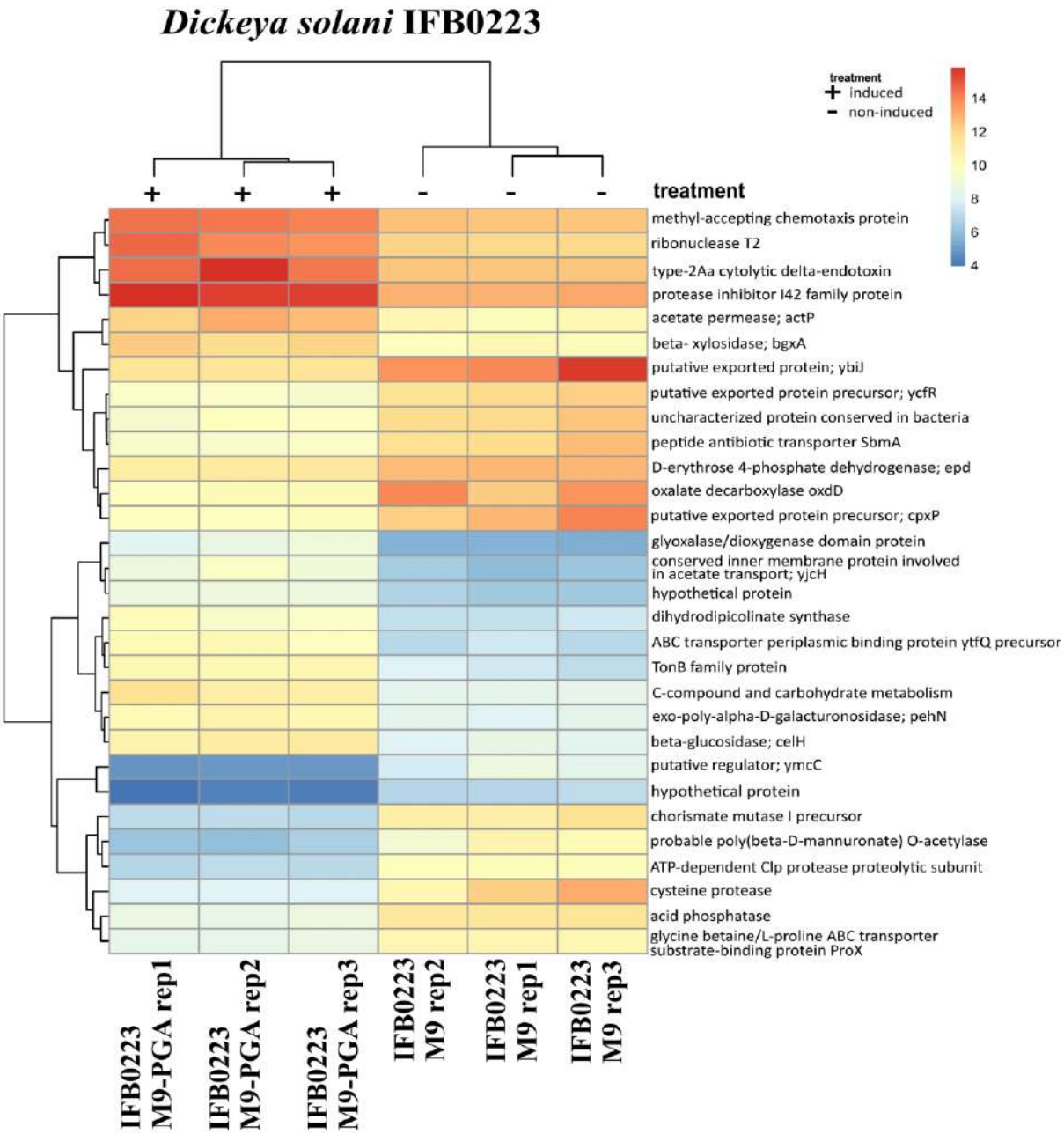

Figure 4. Comparison of the expression values for 30 genes with the lowest adjusted *p-value* for *D. solani* IFB0223 cultivated under induced *vs.* non-induced conditions. Hierarchical clustering was used to generate the heatmap. The scale shows the expmatrix variable, which reflects the expression level of genes transformed using the regularized log technique.

Among the 30 most varying in a statistically significant manner DEGs in IFB0223 between the analyzed conditions, there were several genes potentially associated with the virulence of SRP (Figure 4). One such gene is *cpxP*, encoding a cell-envelope stress modulator involved in protection of bacteria from potentially toxic compounds, that showed lower expression level under the induced

conditions in contrast to the non-induced ones. Another example is a gene coding for a member of the TonB family, outer membrane proteins involved in iron uptake in Gram-negative bacteria, which showed higher expression in the induced conditions compared to the non-induced ones. Also *pehN* gene coding for a polygalacturonase exhibited higher expression in induced compared to non-induced conditions in *D. solani* IFB0223 (Figure 4). As the induced conditions included addition of PGA, the noted higher expression of genes encoding enzymes that release digalacturonides from the pectin chain may be related to availability of this nutrient source.

Notably, several of the genes differentially expressed in *D. solani* IFB0099 under induced compared to non-induced conditions disclosed similar expression pattern in the case of IFB0223 strain. This observation concerns a gene encoding a protease inhibitor I42, which is overexpressed under induced compared to non-induced conditions in both the analysed *D. solani* strains (Figure 3, Figure 4). Even though this gene ranked 4th among most differentially expressed genes in IFB0223 (compared to 1st in IFB0099) (Figure 4), it still exhibited the highest expression level among the 30 most differentially expressed genes. Moreover, the gene encoding the ABC transporter substrate-binding protein exhibited also differential expression between the studied conditions both in IFB0099 and IFB0223 (Figure 3, Figure 4). Though, regarding IFB0099 higher expression of this gene was observed in non-induced conditions, while in the case of IFB0223 in induced conditions.

Post providing insight into 30 most differentially expressed genes in a statistical manner between the induced *vs.* non-induced conditions in two strains of *D. solani*, we moved to examination of changes in the expression of individual genes with special focus on the ones associated with virulence of SRP.

4.4.2 Differential expression of genes encoding major virulence factors of SRP in *D. solani* IFB0223 and IFB0099 cultured under induced *vs.* non-induced conditions

In order to examine deviations in the expression of genes potentially related to the virulence of SRP, differential expression analysis was performed for all genes annotated in the transcriptomes of *D. solani* IFB0099 and IFB0223 (Tables S3-S4). On this basis, a subset of the differentially expressed genes that may contribute to the observed varying virulence levels of *D. solani* IFB0099 and IFB0223 is presented (Tables 2-3).

Table 2. The selected differentially expressed genes in highly virulent *D. solani* IFB0099 in induced compared to non-induced conditions

GeneID[a]	Log2 Fold Change[b]	*p* adj[c]	Gene name[d]	Statistical significance[e]	Gene symbol[f]
Pectinases					
WP_013318983.1	4.66	NA	pectate lyase	NA	*pelD*
WP_013318982.1	3.44	NA	pectate lyase	NA	*pelE*
WP_033111690.1	2.51	1.11E-16	exo-poly-alpha-D-galacturonosidase	S	*pehN*

WP_013318842.1	2.46	NA	endo-pectate lyase	NA	*pelI*
WP_013318985.1	1.92	9.51E-04	pectin esterase	S	*pemA*
WP_013319745.1	1.68	1.40E-03	pectate lyase	S	*pelB*
WP_237703426.1	1.66	2.99E-07	pectate lyase pelL	S	*pelL*
WP_013318984.1	1.57	0.03	pectin acetylesterase	S	*paeY*
WP_013317553.1	1.46	0.01	possible pectinesterase	S	*pemB*
WP_013319746.1	1.08	0.03	pectate lyase	S	*pelC*
WP_013319864.1	0.60	0.71	exo-poly-alpha-D-galacturonosidase precursor	NS	*pehX*
WP_013319863.1	-1.02	0.04	polygalacturonase	NS	*pehW*
WP_013319862.1	-2.85	2.83E-07	exo-poly-alpha-D-galacturonosidase precursor	S	*pehV*
Proteases					
WP_013317949.1	2.35	1.09E-07	secreted protease A precursor	S	*prtA*
WP_013317953.1	1.35	1.43E-05	proteases secretion protein	S	*prtE*
WP_013317954.1	1.17	3.29E-04	alkaline protease secretion ATP-binding protein aprD	S	*prtD*
WP_013317950.1	1.05	0.01	secreted protease C precursor	S	*prtC*
WP_013317956.1	0.68	0.08	secreted protease C precursor	NS	*prtG*
WP_013317952.1	0.57	0.18	outer membrane exporter of proteases	NS	*prtF*
WP_013316020.1	0.21	0.52	serine endoprotease periplasmic	NS	*degS*
WP_013317951.1	0.16	0.83	secreted protease B precursor	NS	*prtB*
WP_013316021.1	-1.93	1.61E-06	serine endoprotease	S	*degQ*
WP_013319029.1	-2.3	8.81E-09	serine endoprotease membrane-associated	S	*degP*
Flagellar proteins					
WP_012769280.1	2.02	8.73E-06	flagellar basal-body rod protein	S	*flgC*
WP_013318510.1	2.01	4.09E-06	flagellar basal-body rod protein	S	*flgB*
WP_013318487.1	1.84	4.62E-06	flagellar hook-basal body complex protein	S	*fliE*
WP_013318509.1	1.83	9.03E-04	flagellar basal-body rod modification protein	S	*flgD*
WP_013318506.1	1.78	1.1E-03	flagellar basal-body rod protein	NS	*flgG*
WP_013318507.1	1.75	6.5E-03	flagellar basal-body rod protein	NS	*flgF*
WP_013318508.1	1,66	5.6E-03	flagellar hook protein	S	*flgE*
WP_013318488.1	1.38	4.49E-04	flagellar M-ring protein	S	*fliF*
WP_013318528.1	1.18	6.12E-05	flagellar transcriptional	S	*flhD*

			activator		
WP_013318489.1	1.12	0.02	flagellar motor switch protein	S	*fliG*
WP_013318515.1	1.09	0.02	predicted flagellar export pore protein	S	*flhA*
WP_013318476.1	1.09	9.81E-04	RNA polymerase sigma factor	S	*fliA*
WP_013318501.1	1.02	0.03	flagellar hook-associated protein	S	*flgL*
WP_013318496.1	1.02	0.04	flagellar motor switch protein	S	*fliN*
WP_013318527.1	0.99	3.43E-03	flagellar transcriptional activator	S	*flhC*
Proteins involved in degradation of KDG					
WP_013318071.1	5.60	7.47E-04	oligogalacturonate specific porin	S	*kdgM*
WP_013317881.1	2.66	2.87E-02	oligogalacturonate specific porin	S	*kdgN*
WP_013318064.1	2.36	3.20E-03	pectin degradation protein	S	*kdgF*
WP_013317813.1	1.21	0.02	2-dehydro-3-deoxyphosphogluconate aldolase	S	*kdgA*
WP_013318085.1	0.62	0.34	DNA-binding transcriptional regulatory protein	NS	*kdgR*
WP_013315827.1	-1.73	NA	2-dehydro-3-deoxygluconate kinase	NA	*kdgK*
Proteins associated with T2SS					
WP_013318773.1	1.49	0.03	necrosis-inducing protein	S	*nipE*
WP_013318805.1	-0.2	0.67	type II secretion system minor pseudopilin	NS	*gspI*
WP_013318341.1	-0.54	0.13	type II secretion system minor pseudopilin	NS	*gspJ*
WP_013318340.1	-0.55	0.27	type II secretion system minor pseudopilin	NS	*gspK*

[a]Unique gene identifier based on the annotation from the genome of *D. dadantii* 3937 (accession number ASM14705v1)

[b]The log2 fold change value indicating the magnitude of deviation in expression of this gene in *Dickeya solani* IFB0099 between the induced compared to non-induced conditions

[c]The adjusted *p*-value, *i.e.* Benjamini-Hochberg corrected *p*-value, accounting for multiple testing and controlling the false discovery rate. 'NA' means not available

[d]Gene name refers to the unique identifier based on the annotation from the genome of *D. dadantii* 3937 (accession number ASM14705v1)

[e]Statistical significance determined on the basis of *p* adj (p adj < 0.05). 'S' refers to a statistically significant difference and 'NS' to lack of statistically significant differences in gene expression between induced compared to non-induced conditions. Additionally 'NA' means not available

[f]Gene symbol based on the annotation from the genome of *D. dadantii* 3937 (accession number ASM14705v1)

Table 3. The selected differentially expressed genes in low virulent *D. solani* IFB0223 under induced compared to non-induced conditions

GeneID[a]	Log2 Fold Change[b]	*p* adj[c]	Gene name[d]	Statistical significance[e]	Gene symbol[f]
Pectinases					
WP_013318983.1	4.20	1.20E-04	pectate lyase	S	*pelD*
WP_013318982.1	2.99	2.24E-07	pectate lyase	S	*pelE*
WP_033111690.1	2.94	4.73E-17	exo-poly-alpha-D-galacturonosidase	S	*pehN*
WP_013318842.1	3.20	0.01	endo-pectate lyase	S	*pelI*
WP_013318985.1	1.79	3.24E-05	pectin esterase	S	*pemA*
WP_013319745.1	3.30	4.61E-06	pectate lyase	S	*pelB*
WP_237703426.1	1.21	0.19	pectate lyase pelL	NS	*pelL*
WP_013318984.1	0.84	0.09	pectin acetylesterase	NS	*paeY*
WP_013317553.1	1.94	4.62E-04	possible pectinesterase	S	*pemB*
WP_013319746.1	2.42	1.40E-03	pectate lyase	S	*pelC*
WP_013319864.1	0.72	0.43	exo-poly-alpha-D-galacturonosidase precursor	NS	*pehX*
WP_013319863.1	-0.37	0.65	polygalacturonase	NS	*pehW*
WP_013319862.1	-1.24	0.16	exo-poly-alpha-D-galacturonosidase precursor	NS	*pehV*
Proteases					
WP_013317949.1	2.43	0.01	secreted protease A precursor	S	*prtA*
WP_013317953.1	0.06	0.94	proteases secretion protein	NS	*prtE*
WP_013317954.1	0.47	0.52	alkaline protease secretion ATP-binding protein aprD	NS	*prtD*
WP_013317950.1	0.14	0.88	secreted protease C precursor	NS	*prtC*
WP_013317956.1	0.07	0.96	secreted protease C precursor	NS	*prtG*
WP_013317952.1	-0.41	0.59	outer membrane exporter of proteases	NS	*prtF*
WP_013316020.1	0.07	0.90	serine endoprotease periplasmic	NS	*degS*
WP_013317951.1	-0.66	0.24	secreted protease B precursor	NS	*prtB*
WP_013316021.1	-2.06	7.86E-05	serine endoprotease	S	*degQ*
WP_013319029.1	-4.81	2.29E-07	serine endoprotease membrane-associated	S	*degP*
Flagellar proteins					
WP_012769280.1	1.69	NA	flagellar basal-body rod protein	NA	*flgC*
WP_013318510.1	1.83	0.07	flagellar basal-body rod protein	S	*flgB*

WP_013318487.1	1.81	0.05	flagellar hook-basal body complex protein	NS	*fliE*
WP_013318509.1	1.55	NA	flagellar basal-body rod modification protein	NA	*flgD*
WP_013318506.1	1.53	0.18	flagellar basal-body rod protein	NS	*flgG*
WP_013318507.1	1.43	0.26	flagellar basal-body rod protein	NS	*flgF*
WP_013318508.1	1.72	NA	flagellar hook protein	NA	*flgE*
WP_013318488.1	1.54	0.07	flagellar M-ring protein	NS	*fliF*
WP_013318528.1	1.74	0.02	flagellar transcriptional activator	S	*flhD*
WP_013318489.1	0.86	0.1	flagellar motor switch protein	NS	*fliG*
WP_013318515.1	0.83	0.03	predicted flagellar export pore protein	S	*flhA*
WP_013318476.1	0.6	0.16	RNA polymerase sigma factor FliA	NS	*fliA*
WP_013318501.1	1.01	0.09	flagellar hook-associated protein	NS	*flgL*
WP_013318496.1	1.0	0.1	flagellar motor switch protein	NS	*fliN*
WP_013318527.1	1.63	0.04	flagellar transcriptional activator	S	*flhC*
Proteins involved in degradation of KDG					
WP_013318071.1	3.77	4.0E-04	oligogalacturonate specific porin	S	*kdgM*
WP_013317881.1	3.77	3.96E-04	oligogalacturonate specific porin	S	*kdgN*
WP_013318064.1	2.01	3.41E-4	pectin degradation protein	S	*kdgF*
WP_013317813.1	0.28	0.67	2-dehydro-3-deoxyphosphogluconate aldolase	NS	*kdgA*
WP_013318085.1	0.44	0.28	DNA-binding transcriptional regulatory protein	NS	*kdgR*
WP_013315827.1	-1.33	0.09	2-dehydro-3-deoxygluconate kinase	NS	*kdgK*
Proteins associated with T2SS					
WP_013318773.1	0.48	0.74	necrosis-inducing protein	NS	*nipE*
WP_013318805.1	-1.15	0.02	type II secretion system minor pseudopilin	S	*gspI*
WP_013318341.1	-1.26	0.03	type II secretion system minor pseudopilin	S	*gspJ*
WP_013318340.1	-1.10	0.11	type II secretion system minor pseudopilin	NS	*gspK*

[a]Unique gene identifier based on the annotation from the genome of *D. dadantii* 3937 (accession number ASM14705v1)

[b]The log2 fold change value indicating the magnitude of deviation in expression of this gene in *Dickeya solani* IFB0099 between the induced compared to non-induced conditions

[c]The adjusted *p*-value, *i.e.* Benjamini-Hochberg corrected *p*-value, accounting for multiple testing and controlling the false discovery rate. 'NA' means not available

[d]Gene name refers to the unique identifier based on the annotation from the genome of *D. dadantii* 3937 (accession number ASM14705v1)

[e]Statistical significance determined on the basis of *p* adj (*p* adj < 0.05). 'S' refers to a statistically significant difference and 'NS' to lack of statistically significant differences in gene expression between induced compared to non-induced conditions. Additionally 'NA' means not available
[f]Gene symbol based on the annotation from the genome of *D. dadantii* 3937 (accession number ASM14705v1)

The genes of potential involvement in the virulence of SRP that also exhibited differential expression in the induced *vs.* non-induced conditions in *D. solani* IFB0099 and IFB0223, could be grouped into the following categories: encoding pectinases for pectin degradation, related to the synthesis and secretion of proteases, encoding flagellar proteins, coding for proteins involved in degradation of KDG and the last one associated with T2SS.

In more detail, the presence of PGA in the medium induced in both *D. solani* IFB0099 and IFB0223 strains overexpression of genes encoding endo-pectate lyases *pelD, pelE, pelI, pelB, pelL, pelC,* pectin acetyl esterase *paeY,* pectin methyl esterases *pemA, pemB* and rhamnogalacturonate lyase *rhiE* (Tables 2-3), which are involved in degradation of an important component of plant cell wall such as pectin chain. Of the above-mentioned genes, *pelD, pelE, pelL* showed higher levels of induction of expression in the presence of PGA in the virulent strain (IFB0099) compared to the low virulent one (IFB0223) as the recorded log2 fold changes for *pelD, pelE, pelL* genes equaled 4.66, 3.44 and 1.66 for IFB0099 (Table 2) and 4.20, 2.99 and 1.21 for strain IFB0223 (Table 3), respectively. Moving to other enzymes playing a role in the catabolism of pectins, pectin acetyl esterase *paeY* in addition to pectin methyl esterases *pemA* and *pemB* revealed higher expression levels in the induced conditions compared to non-induced ones in both the analyzed *D. solani* strains, although differences in the induction rates were noted between the more virulent strain compared to the less virulent one (Tables 2-3). Precisely, the expression levels of *paeY* and *pemA* were boosted to a higher extent in IFB0099 (Log2FC 1.57 and 1.92, respectively) (Tables 2) compared to IFB0223 (Log2FC 0.84 and 1.79, respectively) (Table 3). On the other hand it is worth to acknowledge that the expression of *pemB* gene turned out to be more stimulated in the low virulent strain (log2FC 1.94) (Table 3) than in the virulent one (log2FC 1.46). Passing to exo-polygalacturonases (PehV, PehW, PehX), *pehV, pehW* genes showed reduced expression in both the analyzed *D. solani* strains cultured in the presence of PGA (Tables 2-3). Notably, the expression of these genes was at a much lower level in the case of IFB0099 compared to IFB0223 (log2FC for IFB0223 equals -1.24 and -0.37, while for IFB0099 it is -2.85 and -1.02 in case of *pehV* and *pehW* respectively). At the same time, the changes in expression of *pehX* between the studied conditions were at a comparable level in both strains (Tables 2-3). It is worth to underline that endo-polygalacturonase *pehN*, which also was identified among 30 most significantly expressed genes in IFB0223 (Figure 4), was also revealed to be upregulated in both low and highly virulent *D. solani* if the strains had been cultured with PGA (log2FC for IFB0223 equals 2.94, while for IFB0099 it is 2.51).

Passing to another group of PCWDEs that contributes to the virulence of SRP, among four proteases (PrtG, PrtA, PrtB, PrtC) produced by *Dickeya* spp., all genes, except for *prtA*, that encode

these proteins showed higher expression in IFB0099 compared to IFB0223 strain (Tables 2-3). *PrtA* gene, on the other hand, was revealed to be similarly upregulated in both the analyzed *D. solani* strains (log2FC for IFB0223 equaled 2.43 and for IFB0099 amounted 2.35) (Tables 2-3). Regarding the *prtD, prtE, prtF* genes, they exhibited higher stimulation of expression in the presence of PGA in the virulent strain (IFB0099) compared to the low virulent one (IFB0223) (Tables 2-3), as log2 fold changes recorded for these genes amounted 1.17, 1.35 and 0.57 for strain IFB0099 and 0.47, 0.06 and -0.41 for strain IFB0223, respectively (Tables 2-3). Moreover, the expression of genes encoding serine endoproteases (belonging to the group of Htr proteases) *degQ* and *degP* turned out to be downregulated under induced compared to non-induced conditions in both low and high virulent strains (log2FC for IFB0099 equaled -1.93 and -2.3 respectively and for IFB0223 amounted -2.06 and -4.81 respectively) (Tables 2-3). On the contrary, *degS* gene coding for another serine endoprotease showed a log2 fold change of 0.21 for IFB0099 strain and 0.07 for IFB0223 (Tables 2-3), therefore expression of this gene did not differ notably both between the studied conditions and the included *D. solani* strains. Nonetheless, all investigated genes encoding the Htr proteases showed higher stimulation of expression in the case of IFB0099 strain compared to IFB0223.

Other differences identified between the transcriptomes of IFB0099 and IFB0223 strains cultured under induced or non-induced conditions, are associated with bacterial movement. There were 15 genes identified encoding flagellar proteins, *i.e. flgC, flgB, fliE, flgD, fliG, flgG, flgF, flgE, fliF, flhD, flhA, fliA, flgL, fliN, flhC* of higher expression under induced compared to non-induced conditions in both the analyzed *D. solani* strains. Expression of eight, *i.e. flgC, flgB, flgD, fliG, flgG, flgF, flhA, fliA* out of these 15 genes, turned out to be more efficiently stimulated in the virulent strain (IFB0099) compared to the low virulent one (IFB0223) (Tables 2-3). The remaining seven genes, *i.e. fliE, flgL, fliN, flgE, fliF, flhD* and *flhC*, showed deviations in expression resulting from the presence of PGA at a similar level (*fliE, flgL, fliN*) or increased (*flgE, fliF, flhD, flhC*) in the case of IFB0223 compared to IFB0099.

Moving to genes involved in metabolizing oligogalacturonides, *kdgM* coding for a transmembrane porin from that pathway was revealed to be highly upregulated in the presence of PGA in both IFB0099 and IFB0223 strains, however, a more efficient stimulation of expression was observed in the virulent (log2FC 5.60) than the low virulent strain (log2FC 3.77) (Tables 2-3). Focusing on other genes involved in the metabolism of unsaturated galacturonate, the expression rates of *kdgA* and *kdgF* were boosted in a higher manner in the case of IFB0099 than IFB0223 (with log2FC for IFB0099 being 1.21 and 2.36 and for IFB0223 0.28 and 2.01, respectively) (Tables 2-3). On the other hand, the expression of another gene playing a role in breaking down oligogalacturonides *kdgN* was noted to be upregulated in the presence of PGA in both the analyzed *D. solani* strains, however, IFB0223 exhibited more efficient increase in expression of this gene than IFB0099 (log2FC for IFB0099 equaled 2.66, while for IFB0223 it amounted 3.77) (Tables 2-3). Passing on to the last gene, *kdgK* coding for a protein catalyzing phosphorylation of KDG, this gene showed reduced expression in the presence of PGA in both the analyzed strains, interestingly higher decrease in expression of this

gene was noted for IFB0099 than IFB0223 strain (log2FC for IFB0099 was -1.73, while for IFB0223 it equaled -1.33) (Tables 2-3). The above-mentioned genes are collectively regulated by the repressor KdgR, whose gene showed similar expression under induced conditions compared to non-induced conditions in both the analyzed strains (with log2FC for IFB0099 0.44 and 0.62 for IFB0223) (Tables 2-3).

Last but not least, among the genes that may influence the virulence of SRP, we identified the genes related to T2SS. The expression of its building block *gspJ* was impaired in the presence of PGA in both *D. solani* strains, though the reduction rate was higher in IFB0223 than IFB0099 (log2FC for IFB0099 amounted -0.54 and for IFB0223 equaled -1.26) (Tables 2-3). Additionally, the *gspI* and *gspK* genes encoding other components of T2SS showed similar expression pattern as *gspJ*, as their expression was also decreased under induced conditions to a lower extent in the virulent strain (IFB0099) compared to the low virulent one (IFB0223) (with log2FC for IFB0099 being -0.20 and -0.55 and for IFB0223 -1.15 and -1.10, respectively) (Tables 2-3). Passing to effectors of T2SS, a gene encoding necrosis-inducing protein *nipE*, exhibited higher expression under induced compared to non-induced conditions in both the studied IFB0099 and IFB0223 strains (Tables 2-3). It is worth to stress that we observed higher stimulation of *nipE* expression in the presence of PGA in the investigated virulent strain (IFB0099) compared to the low virulent one (IFB0223) (log2FC for IFB0099 amounted 1.46, while for IFB0223 it was 0.48) (Tables 2-3).

4. DISCUSSION

The previously conducted comparative genomic analyses on 22 *D. solani* strains disclosed overrepresentation of transcription-associated COGs in the accessory and unique pangenome fractions contrarily to the studied core part [12]. A notable focus was put on regulation of gene expression between *D. solani* strains of diverse pathogenic potential. Therefore in this research, DNA methylation analysis of *D. solani* IFB0099 and IFB0223 strains differing significantly in the virulence revealed, to the best of our knowledge, the first genome-wide localizations and types of methylation sites in this phytopathogenic species.

The herein preformed DNA methylation analysis revealed three m6A-type methylation motifs in both *D. solani* IFB0099 and IFB0223, which is consistent with the reports that the m6A-type methylation is most frequently detected sort of DNA methylation within the bacterial kingdom [49]. Before, a linkage was disclosed between DNA adenine methylation and bacterial virulence, while studying human pathogens, for example enterohaemorrhagic *Escherichia coli* O157:H7, *Mycobacterium tuberculosis* or *Streptococcus mutans* [50, 51, 52]. Likewise, associations between DNA methylation and bacterial virulence were suggested for the plant pathogenic bacterium *Ralstonia solancearum* [53]. In that research, both m6A and m4C methylation were recorded at a significantly higher frequency in a low virulence strain compared to a highly virulent *R. solancearum* strain [53]. It is also worth to acknowledge that methylation of the 5'-GATC-3' motif is a prevalent epigenetic modification among bacteria from the Gammaproteobacteria class, comprising the herein studied *D.*

solani strains, due to the activity of the Dam MTases [54]. It was demonstrated in *E. coli* that the lack of the Dam-dependent methylation leads to pleiotropic defects, highlighting crucial importance of the Dam-dependent GATC methylation [55]. Furthermore, Dam was demonstrated to control key genes implicated in various facets of bacterial pathogenesis, for instance, the pyelonephritis-associated pilus (Pap) operon found in uropathogenic *E. coli* (UPEC) [56, 57]. While the herein reported DNA methylation analysis of *D. solani* IFB0099 and IFB0223 did not result in unveiling any obvious differences in the DNA methylation patterns that could explain diverse virulence levels of these strains, it has been revealed that few 5'-GATC-3' motifs show robust methylation across the chromosome and population, which is consistent with previous studies on Dam MTases in the other bacterial species [58].

Subsequently, we showed by pioneer transcriptome profiling of the two *D. solani* strains cultured under conditions mimicking diverse stages of the plant infection process [25, 26, 27], significant changes in the gene expression patterns not only between different growth conditions, but also amongst the *D. solani* strains of various virulence potential. The influence of the medium composition on gene expression was noted in a study conducted by Jiang et al. (2016) [17]. They investigated alterations in the transcriptomes of *D. dadantii* in reaction to the medium composition, both in the exponential and the early stationary growth phase. In the case of medium supplemented with PGA, the numbers of induced and repressed genes in the stationary phase were 682 and 846, respectively ($|FC| > 2$, $FDR < 0.001$), which indicates a larger number of the identified genes than in the here presented work (upregulated genes 546 and 466; downregulated genes 479 and 436 in the case of IFB0099 and IFB0223, respectively). Contrarily, the studies on *D. dadantii* during the early stage of interaction (6 hpi) with *Arabidopsis thaliana* showed 306 upregulated and 269 downregulated genes [59], being lower numbers than in the current work. To sum up, it seems that the numbers of differentially expressed genes depend both on the medium from which the bacteria are collected, as well as on their growth phase or infection stage.

In the subsequently performed transcriptomic analysis we revealed multiple differentially expressed genes between the investigated induced *versus* non-induced conditions, which corresponded to diverse disease progression stages, in both the studied *D. solani* strains of varying virulence potential. It should be highlighted that the key virulence factors associated with pathogenicity of *Dickeya* species include, among others, pectinases [60]. Previous studies have shown that *D. dadantii* 3937 mutant lacking the *pelABCDEZ* genes is significantly impaired in its virulence on various plant species [26, 61, 62]. Here, we observed that genes encoding three endo-pectate lyases (*e.g. pelD, pelE, pelL*) were induced by the presence of PGA and their stimulation of expression was higher in *D. solani* IFB0099 compared to IFB0223. It is worth to consider that in *D. dadantii* 3937 model strain, the expression of *pelA*, *pelD* and *pelE* genes changed differently in response to environmental stimuli relevant to plant infection [60]. In more detail, the expression of *pelD* turned out to be elevated in response to oxidative stress, while the transcription of *pelE* was triggered by osmotic stress [17]. These above-listed responses likely mirror the temporal regulation of these genes during the infection

process [17]. It is worth noticing that the mutants of *D. dadantii* 3937 lacking the *pelL* gene exhibited decreased potency in infecting potato tubers [25]. In particular the activity of PelL seemed crucial during the initial stages of the infection [25]. The current study is in accordance with the latter one as we noted an increased expression of the *pelL* gene in a virulent strain (IFB0099) compared to a low virulent one (IFB0223), despite the fact that the present experimental setup involved bacteria in the stationary growth phase.

Among the DEGs reported in this research, there are also proteases, which break down proteins not only building up plant cell wall, but also the ones involved in host responses to bacterial infection [60]. Here from the four genes coding for proteases (*prtG, prtA, prtB, prtC*) in *Dickeya* spp., *prtG, prtB* and *prtC* exhibited elevated expression in the presence of PGA in highly virulent IFB0099 on the contrary to IFB0223 strain. On the other hand, *prtA* gene turned out to be strongly upregulated under induced conditions in both the herein studied *D. solani* strains. These data are in accordance with Pédron et al. (2018) [59], who observed notably elevated expression of *prtA* and *prtB* (Fold Change: 13.61 and 10.32 respectively) at 24 h compared to 6 h of Arabidopsis thaliana leaf colonization by *D. dadantii* 3937. Even though the influence of proteases on the virulence of *Dickeya* spp. is a controversial topic [11, 27, 63, 64] the herein presented outcomes support studies pointing to contribution of these enzymes to disease symptoms development. In more detail, the results of current transcriptomic profiling are in line with research on *D. dadantii* 3937 and *D. solani* IFB0223 mutants affected in proteases production that at the same time exhibited reduced virulence on saintpaulia or potato, respectively [28, 64]. Contradictive point of view is presented by Dahler et al., (1990) [63], who claimed that mutations in protease genes of *Erwinia chrysanthemi* had no effect on virulence towards plant tissues [63].

Interesting outcomes are also associated with DEGs involved in bacterial movement. We noted higher expression of 14 genes encoding flagellar proteins in both the analysed *D. solani* strains under induced in contrast to non-induced conditions. Notably, the expression of eight (*flgC, flgB, flgD, fliG, flgG, flgF, flhA, fliA*) of these genes showed higher stimulation in the virulent strain *D. solani* IFB0099 compared to the less pathogenic IFB0223. As the addition of PGA simulated more advanced stages of the infection process [11, 22, 25], there is an accordance with former research demonstrating high expression of flagella-related genes during tuber maceration by *Pectobacterium brasiliense* [65]. Notable support for contribution of motility to disease symptoms development was provided by Antunez-Lamas (2009) [66], who showed that mutations in genes *motA* and *cheY*, involved the processes of movement and chemotaxis, impaired the virulence of *D. dadantii* 3937 towards several hosts, including chicory and potato. Also former research by Golanowska et al. (2018) [11] revealed that low virulent *D. solani* IFB0223 is at the same time more adhesive but less motile compared to the virulent IFB0099 strain. Among the herein revealed DEGs that turned out to be stimulated under induced conditions in a more effective manner in IFB0099 strain than IFB0223, there was *fliA* encoding a flagellar sigma factor FliA pointed before as important for the virulence of *D. dadantii* [67]. In more detail, a *D. dadantii fliA* null mutant exhibited in the research of Jahn et al., (2008) [67]

diminished virulence, reduced adherence to plant tissue, decreased Pel activity in addition to impaired biofilm formation. The herein revealed boosted expression of *fliA* in the presence of PGA in IFB0099 compared to IFB0223, finds agreement with the reduced motility of IFB0223 in juxtaposition to IFB0099 described previously by Golanowska et al. (2018) [11]. The latter observation supports hypothesis on the contribution of flagellar proteins to differences in virulence between the studied *D. solani* strains of various pathogenic potential. However, the expression of *flhC* and *flhD* genes, coding for the components of a FlhD/FlhC complex (an activator of the transcription of flagellar operons) [68], turned out to be more efficiently stimulated in IFB0223 compared to IFB0099 under non-induced conditions. Altogether, further research is needed to clarify individual contributions of the flagella-related genes into the pathogenicity of *D. solani* strains differing in their pathogenic potential.

This research also revealed DEGs, which are involved in the catabolism of oligogalacturonides. We showed that the expression of a gene coding for a transmembrane porin KdgM, engaged in transferring extracellular unsaturated oligogalacturonides generated from pectin degradation by pectin lyases into periplasmatic space [69], was more efficiently stimulated by PGA in the virulent *D. solani* IFB0099 than the low virulent strain IFB0223 strain. Also some other genes engaged in decomposition of unsaturated galacturonates, *i.e. kdgA* and *kdgF*, turned out to be expressed in a more efficient manner in *D. solani* IFB0099 than IFB0223 post induction with PGA. There is a support for putative roles of the constituents of a KDG pathway in the virulence of *D. solani*, as formerly mutants in *kdg* genes from a closely related species *D. dadantii* turned out to be less competitive in infecting chicory leaves than the wild type strain [70]. Interestingly, *kdgN* gene, which was here revealed to be stimulated in both the analysed *D. solani* strains by the presence of PGA, showed higher stimulation of expression in IFB0223 than IFB0099. It is worth mentioning that this gene tends to be down-regulated during early stages of infection (8 hpi) when the pathogen penetrates into plant leaves tissue as shown in *D. dadantii* [71]. As the herein studied bacterial populations were in a stationary phase of growth, the observed stimulation of expression of *kdgN* is in agreement with the literature data. The case of another gene involved in assimilation of oligogalacturonides is puzzling as the expression of *kdgK* was decreased in the presence of PGA in both the analyzed strains in spite of the former reports on induction of expression of this gene 24 h post infection of Arabidopsis [59]. The latter discrepancy might be associated with the fact that this study involved 48h-grown bacterial cultures, in contrast to Pedron et al. (2018) [59] experiments performed 24h after inoculation of plant tissue with bacteria. It has been reported before that the genes engaged in oligogalacturonide catabolism are located in diverse regions of bacterial genome and are subjected to the control of KdgR repressor [22]. Here, the expression of gene coding for this repressor was slightly stimulated by the presence of PGA in both *D. solani* strains, but it is worth to consider that formation of KDG at the end of the discussed pathway shall anyway lead to inactivation of KdgR [22].

It should be underlined that pectinases are secreted *via* T2SS by the members of the *Dickeya* genus [27]. In the current study we observed that the gene *gspJ*, encoding a T2SS pseudopilin, shows lower expression under induced compared to non-induced conditions in both the analysed *D. solani* strains. Nonetheless, the expression of this gene was decreased to a lesser extent in the highly virulent IFB0099 strain compared to the low virulent IFB0223. Furthermore, the genes coding for GspI and GspK, which form a complex with GspJ [72] enabling the transport of proteins across the outer membrane, also exhibited lower decrease in expression in the virulent strain compared to the low virulent one under the applied induced conditions. We assume that diverse expression rates recorded for the genes coding for the components of T2SS might be associated with diverse virulence levels between *D. solani* strains, as before *gspJ* was reported for instance to be required for pathogenicity in *Burkholderia cenocepacia* [73]. Further linkage with T2SS-dependent bacterial virulence concerns one of the herein revealed DEGs encoding a necrosis-inducing protein NipE [74]. In the current study, *nipE* gene showed higher stimulation of expression in the presence of PGA in IFB0099 strain than in the case of IFB0223. This outcome is in accordance with the former research of Mattinen et al. (2004) [75] that disclosed decreased efficacy of a *Pectobacterium parmentieri* SCC3193 *nipE* mutant in macerating potato tubers.

5. CONCLUSIONS

To the best of our knowledge, this is the first study on methylomes and transcriptomes of *D. solani* strains differing in the virulence, which aims to elucidate the factors influencing their distinct pathogenic capabilities against economically vital crops. Despite the lack of differences in DNA methylation, there were noted variations in the expressions of genes related to virulence of SRP that aroused our curiosity. The conducted transcriptomic profiling provided some indications on putative contribution of genes encoding pectin lyases, associated with synthesis and secretion of proteases, encoding constituents of flagella, coding for proteins engaged in degradation of KDG or linked with T2SS to diverse pathogenic potential of *D. solani* IFB0099 and IFB0223. As a result several candidate genes have been revealed that should be further studied in the future by the means of constructing deletion mutants and their subsequent compensation to obtain clear answers on the roles of the herein pointed DEGs in the virulence of economically significant, necrotrophic phytopathogen *D. solani*.

Availability of data and materials

The RNA-Seq data for *Dickeya solani* strains of different levels of virulence are publicly available in the Sequence Read Archive repository at http://www.ncbi.nlm.nih.gov/bioproject/944547. All data generated or analyzed during this study are included in this published article (or in its supplementary information files).

Abbreviations

DEGs: differentially expressed genes
KDG: 2-keto-3-deoxygluconate
MLST: multilocus sequence typing
MTases: methyltransferases
PCA: principal component analysis
PCWDEs: plant cell wall degrading enzymes
PFGE: pulsed-field gel electrophoresis

PGA: polygalacturonic acid
REP PCR: repetitive sequence-based PCR
RSEM: RNA-Seq by expectation-maximization
SRP: Soft Rot *Pectobacteriaceae*
TSA: tryptone soya agar medium
VNTR: variable number of tandem repeats

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Author Contributions
E. L., A. M-P and W. B-W. conceived and designed all experiments. W. B-W. cultured bacteria, isolated RNA and performed bioinformatic analysis. G. C. D. performed methylation analysis. W. B-W, A. M., G. C. D. took part in the discussion on the selection of appropriate bioinformatics tools for visualization of RNA-Seq data. W. B-W. visualized the collected data. W. B-W, A. M-P., A. M., E. L., G. C. D. took part in the discussion on the collected data. W. B-W. wrote the first version of this manuscript. W. B-W, A. M-P., A. M., G. C. D., E. L. prepared the final version of this manuscript that has been accepted by all the other authors. E. L. acquired funding for this study.

Ethics declarations:

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Supplementary information

Search for foundations of virulence in an economically significant plant pathogen *Dickeya solani* *via* transcriptomic profiling

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Table S1. The details on the processing of RNA-Seq reads for *D. solani* strains (IFB0099, IFB0223) under the two applied conditions, each involving three biological replicates

Sample name	Biological repetition	Subset numer	Input Read Pairs	Output Read Pairs	Both Surviving [%]
***D. solani* IFB0099 non-induced**	1	001	8863683	8387082	94.62%
		002	8572849	8037143	93.75%
		003	8737830	8242846	94.34%
		004	8706143	8175496	93.90%
	2	001	9089397	8566664	94.25%
		002	8752241	8159873	93.23%
		003	8952282	8410220	93.94%
		004	8916325	8325991	93.38%
	3	001	8654938	8170970	94.41%
		002	8330113	7793987	93.56%
		003	8434294	7949182	94.25%
		004	8502874	7969619	93.73%
***D. solani* IFB0099 induced**	1	001	9501703	8989718	94.61%
		002	9143407	8579218	93.83%
		003	9261609	8748500	94.46%
		004	9323288	8761726	93.98%
	2	001	8896261	8419982	94.65%
		002	8573073	8031292	93.68%
		003	8761896	8265109	94.33%
		004	8724918	8185783	93.82%
	3	001	8982358	8478702	94.39%
		002	8654677	8076651	93.32%
		003	8774862	8262382	94.16%
		004	8502874	7969619	93.73%
***D. solani* IFB223 non-induced**	1	001	9451696	8913213	94.30%
		002	9114694	8503827	93.30%
		003	9246206	8698386	94.08%
		004	9259374	8655706	93.48%
	2	001	9574797	9054129	94.56%
		002	9217006	8644075	93.78%
		003	9382714	8848064	94.30%
		004	9422168	8851700	93.95%
	3	001	8643789	8145996	94.24%
		002	8338114	7776628	93.27%
		003	8434501	7934538	94.07%
		004	8499191	7943169	93.46%
		001	9172702	8657029	94.38%
		002	8829992	8248389	93.41%

***D. solani* IFB223 induced**	1	003	8985579	8457077	94.12%
		004	8982147	8404973	93.57%
	2	001	8917750	8434489	94.58%
		002	8620539	8070216	93.62%
		003	8782081	8280821	94.29%
		004	8757371	8215832	93.82%
	3	001	9529883	9034917	94.81%
		002	9145505	8596221	93.99%
		003	9330180	8822938	94.56%
		004	9349676	8800422	94.13%

Table S2. Summary of mapping the RNA-seq reads to the reference genome of *D. solani* IPO 2222

Sample name	**Biological repetition**	**No. of total paired-end reads**	**% of overall alignment rate**	**No. of reads aligned concordantly:**		
				0 times	**>1 times**	**exactly 1 time**
***D. solani* IFB0099 non-induced**	1	33 428 797	99.89%	156 964	4 416 952	28 268 651
	2	33 462 748	99.88%	130 663	7 082 490	26 249 595
	3	31 883 758	99.84%	173 760	8 539 938	23 170 060
***D. solani* IFB0099 induced**	1	35 079 162	99.88%	163 500	8 512 951	26 402 711
	2	32 902 166	99.87%	191 712	4 089 555	28 620 899
	3	33 057 745	99.83%	182 768	8 925 415	23 949 562
***D. solani* IFB223 non-induced**	1	34 771 132	99.89%	138 562	7 506 033	27 126 537
	2	35 397 968	99.90%	175 803	3 616 314	31 605 851
	3	31 800 331	99.81%	151 441	10 530 128	21 118 762
***D. solani* IFB223 induced**	1	33 767 468	99.69%	170 778	6 954 603	26 642 087
	2	33 001 358	99.90%	171 973	4 897 000	27 932 385
	3	35 254 498	99.90%	185 984	6 798 126	28 270 388

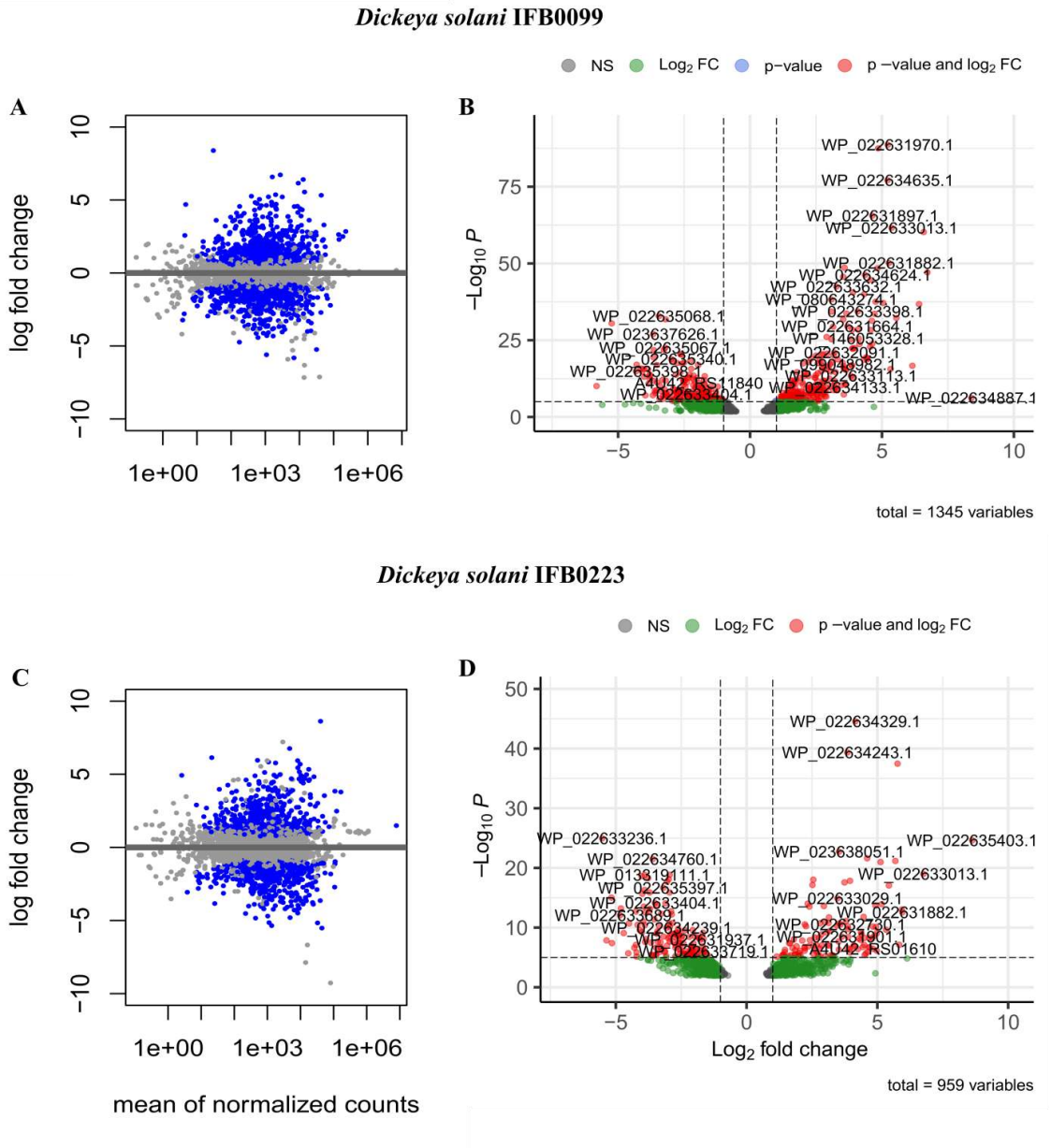

Figure S1. Visualization of the differentially expressed genes in *D. solani* IFB0099 and IFB0223 cultured under induced compared to non-induced conditions

Table S3 All differentially expressed genes in highly virulent *D. solani* IFB0099 in induced compared to non-induced conditions

Table S4 All differentially expressed genes in low virulent *D. solani* IFB0223 in induced compared to non-induced conditions

Table S3 and Table S4 is available at the following link: https://drive.google.com/drive/folders/1zOmq7j0jDBK26QKAzX4RKOiSjUm4NFy9?fbclid=IwAR0zA95jQlbxdXThSVYUKs1k62SRzLAwWG-17XiwIvCiBqizELR5yLuM_M4

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AUTHOR CONTRIBUTION STATEMENT

I declare that in the manuscript:

"Search for foundations of virulence in an economically significant plant pathogen *Dickeya solani* via transcriptomic profiling"

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my contribution involved conceived and designed all experiments. Moreover I cultured bacteria and isolated RNA. Therefore, I performed bioinformatic analysis and visualized the collected RNA-Seq data. Additionally I took part in the discussion on the collected data. I prepared first version of this manuscript and corrected it according to the co-authors' suggestions.

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my involvement included participation in planning of the described experiments, corrections of the first version of the manuscript and discussion of the research results.

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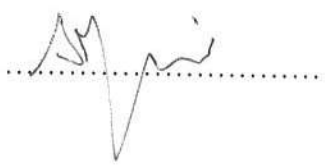
my contribution involved in the discussion on the selection of appropriate bioinformatics tools for visualization of RNA-Seq data. Moreover, I took part in the discussion on the collected data and provided correction to the first version of the manuscript.



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AUTHOR CONTRIBUTION STATEMENT

I, Dr. George diCenzo, declare that in the manuscript:

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My contribution involved: (i) discussion of the selection of appropriate bioinformatics tools for visualization of RNA-seq data; (ii) performing a DNA methylation analysis; (iii) taking part in discussion of the collected data; and (iv) and contributing to revision of first version of the manuscript.

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I declare that, I was responsible for co-conceptualization of the project and designed experiments. I participated in the discussion of the results as well as work preparation on the final version of the manuscript.

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Manuscript No. 2

Differences in the constituents of bacterial microbiota in relation to soil suppressiveness towards potato blackleg and soft rot diseases

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submitted for publication

Differences in the constituents of bacterial microbiota in relation to soil suppressiveness towards potato blackleg and soft rot diseases

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Abstract

The presence of bacteria from the *Dickeya* spp. and *Pectobacterium* spp. in farmlands leads to global crop losses of over $420 million annually. In recent years, scientists have started to suspect that the development of disease symptoms in crops is related to soil suppressiveness. Here, we investigated whether the progression of the *Dickeya* spp. - and *Pectobacterium* spp. - driven infections could be associated with the composition of the soil bacterial microbiota.

The presented analyses indicated that the tested soils suppressive and non-suppressive towards potato blackleg and soft rot diseases had similar physicochemical features. Microbial cultures and molecular diagnostics-based approaches enabled the identification of several isolates of *Pectobacterium* spp. originating from the non-suppressive soil. No isolates of bacteria from *Pectobacterium* and *Dickeya* spp. have been acquired from the field with suppressive soil. In addition, 16S rRNA gene amplicon sequencing revealed differences between the soil bacterial microbiota of suppressive and non-suppressive soils. In particular, bacteria from genera: *Bacillus*, *Acidobacterium* and *Gaiella* turned out to be more abundant in the suppressive than the non-suppressive soil samples.

The herein presented case study, for the first time indicates that the soil bacterial microbiota vary between soils suppressive and non-suppressive towards soft rot *Pectobacteriaceae*-caused infections.

1. Introduction

Pectinolytic bacteria from the *Dickeya* spp. and *Pectobacterium* spp., referred to as Soft Rot *Pectobacteriaceae* (SRP), currently comprise 13 *Dickeya* and 21 *Pectobacterium* species [1-3]. These phytopathogens cause the symptoms of soft rot on potato or other vegetables and ornamental plants as

well as blackleg on potato, cabbage, corn and other economically important plants [2, 4]. The blackleg symptoms can be recognized by blackening of the base of the shoot, which often leads to wilting of the whole plant. Soft rot, on the other hand, is manifested by maceration of the internal tissue of vegetative organs. The global spread of *Dickeya* and *Pectobacterium* spp. results not only in significant yield, but also increasing economic losses [2, 4]. In particular, the presence of SRP in farmlands leads to crop losses reaching up to $420 million annually [5], with the highest economic impact on the potato production sector [6]. In Europe alone, phytopathogens from *Dickeya* spp. and *Pectobacterium* spp. triggered approx. €46 million damage on potato plantations [7]. Moreover, 32%, 43% and 25% of these losses were associated with seed, table and processing potatoes, respectively [7].

Despite the affected food security, widespread presence and expanding geographical range of SRP, there are currently no effective plant protection measures against pectinolytic phytopathogens [6]. Notably, different control approaches relying either on physical, chemical or biological methods have been tested [4], but the number of these methods is scarce and their potency is too low to effectively prevent the spread of these microorganisms in the environment. To date, screening of potato seed lots for the presence of *Dickeya* and *Pectobacterium* spp. has been regarded as the most efficacious crop protection method [6].

Previous studies associated the spread of *Dickeya* and *Pectobacterium* spp. not only with the shipment of latently infected seed potatoes, but also the transfer of contaminated soil [2]. Indeed, SRP are able to retain viability in soil from 1 week to 6 months, depending on the encountered environmental conditions [2]. The ability of these bacteria to survive in soil tends to be species-specific. Most of the studied *Pectobacterium* spp. are capable of surviving in soil for up to several months, while *Dickeya* spp., *e.g. Dickeya dianthicola* strains, are generally regarded as seed-borne pathogens [8]. For instance, *Pectobacterium* spp. isolates were able to survive in soil for approx. two months [9]. At the same time, *Dickeya* spp. remained viable in soil for up to seven days at 6°C and 50% soil moisture [10]. Several factors, like temperature, moisture and the pH of the soil, have an influence on the persistence of SPR in soil [11]. Microbiological analyses of the soil collected in Australia one year before planting of potatoes showed that 25% of the samples contained *Dickeya* or *Pectobacterium* spp. [12]. Among these bacteria, the most prevalent species has been *Pectobacterium carotovorum*, accounting for 93% of the isolates. On the contrary, *Pectobacterium atrosepticum* was found only in 5.6% of the samples. On the other hand, *Dickeya* spp. strains have been not frequently detected in the collected soil as they equalled just about 2% of the total number of identified strains [13-14]. Pectinolytic bacteria that survive in soil can then penetrate plant tissue through mechanical damages or wounds, leading to colonization of the intercellular spaces of the roots prior to invading into the xylem.

Recently, soil suppressiveness against diseases caused by phytopathogens has been linked with interaction between natural soil microbiota and the pathogen [15], in contrast to former suggestions of a dominant impact of abiotic environmental factors [16]. Suppressive soil according

to its microbiological composition, is characterized either by the absence of a certain phytopathogen or its incapability to develop disease symptoms on a susceptible plant host [17]. On the other hand, the microbiota of non-suppressive soil favours the development and progression of disease symptoms on plants. So far, there were several reports on bacterial taxa identified in suppressive soils towards plant diseases. [18-19]. For instance, soil containing bacteria from the genera *Streptomyces*, *Pseudomonas* and *Bacillus* was described to suppress *Fusarium* wilts [18]. Furthermore, the soil-born bacterial pathogen *Ralstonia solanacearum*, which is responsible for bacterial wilt of tomato, was noted to trigger less devastating disease symptoms in soil containing high diversity bacterial communities enriched with bacteria from the genera *Olivibacter*, *Flavihumibacter* and *Flavobacterium* [19].

Importantly, soil suppressiveness has been divided into two subtypes. General suppression is associated with the microbial diversity. In turn, specific suppression is characterized by the occurrence of a single or selected groups/species of microorganisms. Weller et al. (2002) hypothesized that in the case of specific suppression, the microbiota of interest may be transferable between diverse soil types [17]. For example, sterilization and inoculation of four agricultural soils with beneficial bacteria showed that the bacterial microbiome of soil plays a significant role in the suppressive phenotype towards wheat root disease caused by *Fusarium culmorum* [20]. Modern bacterial community profiling methods offer an opportunity to develop more precise views on the structural diversity of the microbiota by allowing for the detection and identification of non-culturable bacteria [21].

The goal of this study was to evaluate if there is a correlation between the incidence of soft rot and blackleg diseases in the fields with the bacterial composition of the so-assigned suppressive and non-suppressive soils towards *Dickeya* spp. and *Pectobacterium* spp. For this reason we identified bacterial taxa by 16S rRNA gene amplicon sequencing within the microbiota of the collected soils [13-14, 22]. Furthermore, classical culture and molecular diagnostics based methods were implemented for detection of *Dickeya* and *Pectobacterium* spp. in the investigated soil samples. As a result, we report bacterial taxa, whose members are present in different abundances between the soils collected from potato fields of diverse incidences of soft rot and blackleg diseases, opening a future perspective for novel biological control measures, involving development of artificial complex microbial consortia.

2. Results and Discussion

2.1 The incidence of blackleg and soft rot on the studied potato farmlands

Regarding the field located in Siemysl and Bonin, the collected soil has been defined as clay according to the classification of the Polish Soil Science Society: Grading classification of soils and mineral formations. In the potato field located in Siemysl, there were no blackleg symptoms identified throughout the entire growing season of 2021 (Table 1). On this basis, this field was selected as containing suppressive soil for further analyses. In the case of non-suppressive soil, collected from the field in Bonin, the incidence of blackleg was estimated at 2% in July and subsequently increased to

20% in the month of the harvest, *i.e.* August. Concerning the disease incidence rate noted during the previous five years, no blackleg or soft rot symptoms were observed on plants growing in the suppressive soil at the Siemysl site, in contrast to the non-suppressive one in Bonin village that promoted development of potato blackleg in addition to other plant diseases.

Table 1. Characteristics of the selected potato farmlands

		Field with suppressive soil		**Field with non-suppressive soil**	
Field location		Siemysl village		Bonin village	
Blackleg disease incidence		July	0%	July	2%
		August	0%	August	20%
Soil type*		clay		clay	
Applied pesticides		insecticides, herbicides, fungicides		insecticides, herbicides, fungicides	
Applied fertilizers	mineral fertilizers	NPK		NPK	
	natural fertilizers	not used		aftercrop	
Diseases reported in the previous 5 years		no reports		blackleg, potato blight, early blight, rhizoctonia, anthracnose	
Origin of the seed potatoes		own material, class A		classified material, class A	
		cultivar Rosetta		cultivar Denar	

*Polish Soil Science Society: Grading classification of soils and mineral formations.

As cropping and management practices are important factors influencing the suppression of plant pathogens [23], surveys on fertilizers and pesticides applied throughout the growing season on the fields of interest were performed (Table 1). Both potato fields were amended with the same types of pesticides and mineral fertilizers. The only difference between the applied agricultural practices involved the dispersion of an aftercrop in the field located in Bonin, as opposed to the lack of this natural fertilization in the farmlands of Siemysl. It was concluded that the agronomic practices were unlikely to explain the differences in the disease incidence between these fields.

Adjusting the fertilization practices has the potential to decrease the occurrence of blackleg and soft rot diseases. A research study incorporating calcium nitrate as a mineral fertilizer demonstrated that a supply of 250 kg/ha (with 19% Ca content) resulted in an average reduction of 20% in blackleg symptoms, with a maximum reduction of 50% [24]. In turn, the use of aftercrop in agriculture increases the biological activity and fertility of the soil and provides minerals – mainly nitrogen. In this context, the research reported by Dubois Gill et al. (2014) seems interesting, showing that nitrogen input, along with soil organic matter content, accounts for 9% of the variability in the blackleg symptom expression observed in the field [25]. Moreover, a high nitrogen supply was found to elevate nitrate levels in the progeny tubers, as demonstrated in the study by Maltas et al. (2018) [26]. Theoretically, this increase in nitrate content could facilitate the development of soft rot in the progeny tubers post-harvest, as soft rot pathogens (SRP) have the capacity to utilize nitrates both as a nitrogen source and an electron acceptor, as previously shown by Smid et al. (1993) [27].

2.2 Physicochemical characteristics of the collected soil samples

To exclude that the suppressiveness of the soil towards blackleg and soft rot diseases was simply linked with differences in the physicochemical properties of these soils, the pH, salinity, organic matter, and the contents of microelements and macroelements, were analysed (Table 2). Temperature, moisture and pH have been previously shown to influence the survival of SRP in soil [28]. In the current study, the suppressive and non-suppressive soils exhibited pH values of 6.1 and 6.4, respectively, allowing for the classification of these soils as "slightly acidic" (pH 5.5 ÷ 6.5) according to the Polish Soil Science Society. Moreover, the moisture of both suppressive and non-suppressive soils was approximately equal (Table 2). Likewise, the temperature at each site was alike when the soil samples were collected. Altogether, the relative lack of differences in pH, moisture, and temperature between the suppressive and non-suppressive soils suggests that these parameters cannot explain the observed deviations in the disease incidence.

Table 2. Physicochemical features of the soil sampled from the investigated potato fields

Feature	Suppressive soil	Non-suppressive soil
Soil moisture [%]	5.42 ± 1.2	5.31 ± 1.47
Salinity NaCl [g/dm^3]	0.26 ± 0.09	0.33 ± 0.08
N-NO_3 [mg/dm^3]	37.4 ± 20.45	41.8 ± 4.65
Cl [mg/dm^3]	46.4 ± 15.64	54.6 ± 6.35
P [mg/dm^3]	42.4 ± 8.87	42.06 ± 4.53
K [mg/dm^3]	107.83 ± 17.14	88.8 ± 10.12
Ca [mg/dm^3]	684 ± 38.27	854.3 ± 127.92

Na [mg/dm^3]	<15	<15
Mg [mg/dm^3]	184.3 ± 23.89	50.9 ± 9.89*
B [mg/dm^3]	0.33 ± 0.05	0.3 ± 0.08
Cu [mg/dm^3]	0.7 ± 0.08	0.83 ± 0.12
Zn [mg/dm^3]	7.2 ± 2.33	4.1 ± 0.46
Mn [mg/dm^3]	7.1 ± 1.34	3.1 ± 1.34*
Fe [mg/dm^3]	59.6 ± 8.7	46.73 ± 9.45
Organic C [% dry weight]	4.5 ± 0.14	1.37 ± 0.12**
Organic substance [% dry weight]	7.81 ± 0.25	2.37 ± 0.2**

The statistically significant differences according to the t-student test are marked as follows: *$p < 0.05$; **$p < 0.005$.

Among the studied microelements and macroelements, the iron availability seems worth investigating as these ions have been previously associated with the severity of the disease symptoms caused by pectinolytic bacteria [29]. In more detail, *Pectobacterium* and *Dickeya* spp. produce and secrete siderophores, molecules capable of forming complexes with iron, which contribute to the overall pathogenicity of these microorganisms [29]. Here, though, the differences in the iron contents between suppressive and non-suppressive soils turned out to be statistically insignificant.

On the other hand, some deviations were noted in the other examined mineral components (Table 2). A statistically significant difference was observed in the contents of Mg and Mn elements. The suppressive soil contained three times more Mg and twice as much Mn compared to the non-suppressive soil. The previous studies showed that Mn ions inhibit the production of pectin lyases, one of the most important virulence factors of *Dickeya* and *Pectobacterium* spp. [30]. On the other hand, Mg assures greater resistance of the older plant tissues to the necrotrophic pathogens, as this constituent of the middle lamella together with Ca boosts the resistance of pectic chains to the action of pectolytic enzymes. Moreover, elevated Mg levels in tubers were discovered to be associated with reduced occurrences of soft rot, as observed in the study by McGuire and Kelman (1986), and also with decreased stem rot incidence, as indicated by Bain et al. (1996) [31-32]. However, the impact of Mg on rot prevention was not as pronounced as that of equivalent amounts of calcium (Ca). These experimental findings align with the results of Dubois Gill et al. (2014), who demonstrated that increased Mg input could effectively delay blackleg disease outbreaks [25]. Based on this data we can assume that the higher concentration of Mg in the suppressive soil might contribute to the observed lack of disease symptoms in this potato field.

Interestingly, the examined suppressive soil contained about twice as much organics as the non-suppressive soil, and the obtained results were statistically significant. Ficke et al. (1973) provided evidence that the enrichment of soil with organic compounds extended the survival of *P. atrosepticum* [33]. Thus, it might be hypothesized that even if pectinolytic bacteria survived longer in the suppressive soil in contrast to the non-suppressive soil, their persistence did not lead to the increased disease incidence due to other environmental or biotic factors.

It is worth acknowledging that out of 13 examined physicochemical features, solely the above-discussed four differed in a statistically significant manner between the suppressive and non-suppressive soil types. Thus, there was enough support to search for other differences between suppressive and non-suppressive soils towards blackleg and soft rot symptoms, this time with special focus on the composition of the soil microbiome.

2.3 The occurrence of *Dickeya* and *Pectobacterium* spp. on the studied potato plantations

Microbiological analysis of the suppressive and non-suppressive soils and potato tubers collected from the studied fields involved cultures of the diluted homogenates on a CVP medium in addition to the PCR-based identification to the species level of the SRP strains. As shown in Table 3, four *Pectobacterium* spp. isolates were identified in the non-suppressive soil (Bonin field), whereas no *Dickeya* and *Pectobacterium* spp. were detected in the case of suppressive soil collected from the field in Siemysl. Additionally, ten isolates of *P. carotovorum*/*P. parmentieri* were detected in young potato tubers harvested from the non-suppressive soil. Likewise, six additional isolates of *Pectobacterium* spp. were identified in the tubers grown in the non-suppressive soil. On the other hand, no pectinolytic *Pectobacteriaceae* were detected in the young potato tubers harvested from the field with suppressive soil.

Table 3. The pectinolytic isolates identified in the samples collected from the investigated potato fields

Soil characteristics	Sample type	No. of detected isolates	Genus/species of the detected isolates
Suppressive soil	soil	not detected	-
	tuber	not detected	-
Non-suppressive soil	soil	4 isolates	*Pectobacterium* spp.
	tuber	10 isolates	*P. carotovorum/ P. parmentieri*
		6 isolates	*Pectobacterium* spp.

The presence of pectinolytic bacteria from the *Pectobacterium* spp. in the potato field in Bonin (with a total of 20 acquired *Pectobacterium* spp. isolates) and the absence of such strains in the farmland in Siemysl is in agreement with the data collected during the long-term monitoring of these potato fields performed by our research group [13-14, 22]. The herein presented data indicate that *Pectobacterium* spp. were detected only in the potato field in Bonin and that a significantly higher number of these isolates was acquired from potato tubers than from the soil. This observation is most likely associated with a higher survival rate of bacteria from the *Dickeya* spp. and *Pectobcaterium* spp. in plant tissues than in the less favourable soil environment. SRP can be isolated from the post-harvest plant residues that provide a suitable environment for the growth and multiplication of bacteria [34]. The latter remark is in agreement with the recent data of Toth et al. (2021) indicating that SRP populations are often detected after potato harvesting, but then decrease overwinter to very low levels [2].

2.4 The numbers of ASVs and species diversity in the suppressive and non-suppressive soils samples

16S rRNA gene amplicon sequencing of five suppressive and five non-suppressive soil samples collected from the potato fields of interest was performed. The number of paired raw reads generated for each sample ranged from 91,124 to 101,293. An average of 76% of all the readings passed the quality control threshold (Table S1). After the clustering step, the number of the determined ASVs enclosed in the range of 767 – 1024 ASVs per sample (Table S1). The number of ASVs computed for suppressive and non-suppressive soil samples are comparable. The rarefaction curves obtained from the ASVs reached a plateau for all the included samples (Fig. S1), indicating a satisfactory sequencing depth of each sample for studying bacterial diversity between suppressive and non-suppressive soils.

In order to get a better insight into the microbial diversity of soils differing in their level of suppressiveness towards SRP, alpha- and beta-diversities of the bacterial microbiome were calculated. At first, the alpha diversity estimates were computed using Evenness (Pielou's index) and Shannon tests. No significant differences between the alpha-diversity of suppressive and non-suppressive soil samples were detected ($p > 0.05$; Kruskal-Wallis test) (Fig. S2A, S2B).

PCoA and PERMANOVA were used to infer similarities among the sampled microbiota and in relation to soil suppressiveness. PCoA calculations using the Bray-Curtis distance matrix were performed to determine the relatedness of suppressive and non-suppressive soil samples. The PCoA plot revealed separate grouping of suppressive and non-suppressive soil samples (Fig. 1A), suggesting some structural differences between the studied types of soil suppressiveness. PERMANOVA-based analysis pointed to statistical significance of the observed deviations according to pseudo-F at $p = 0.01$ (Fig. 1B). The obtained data suggest that the bacterial communities of the suppressive and non-suppressive soils differ. Moreover, the suppressive soil shows greater diversity of bacteria than the non-suppressive soil. This is in agreement with the data showing that a high diversity of the bacterial

community in soil often has a positive effect on the suppression of the growth of pathogens [19]. Additionally, it was presented that microbial diversity influenced the ability of *R. solanacearum* to induce wilting disease in tomato [35]. However, this relationship was not observed in the case of *R. solanacearum* infection on potatoes [36].

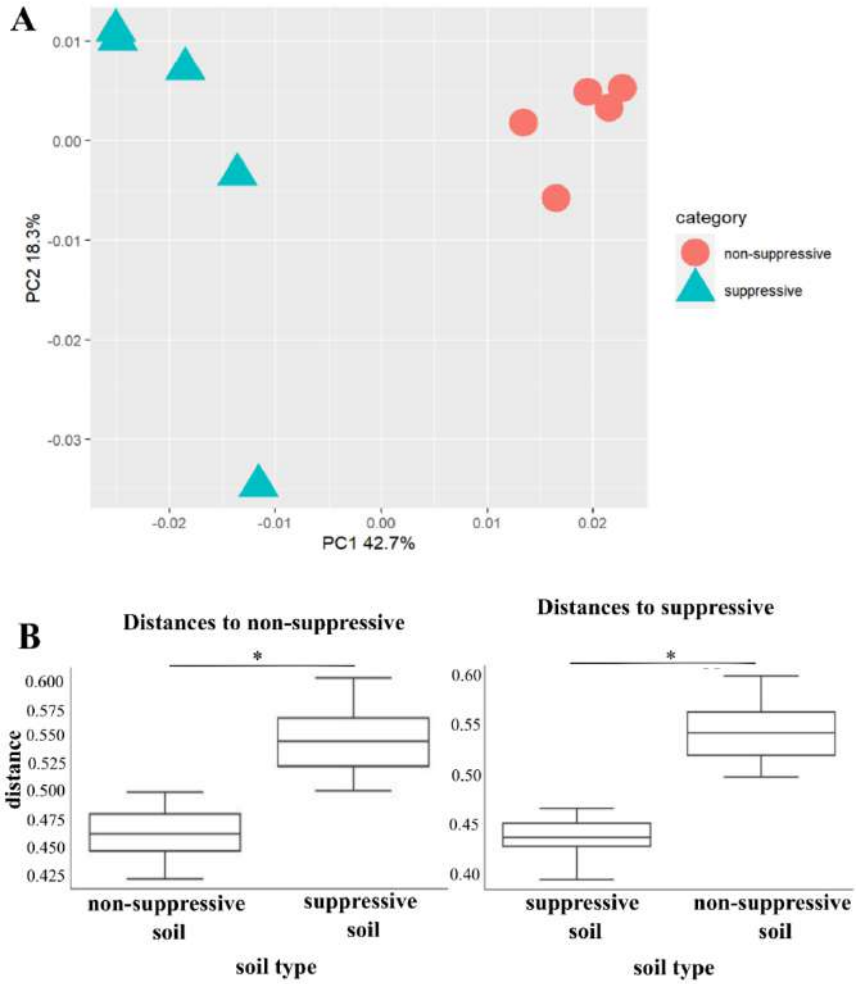

Figure 1. Beta-diversity indices of the suppressive and non-suppressive soil samples. A. Principal coordinates analysis (PcoA) using the Bray–Curtis distance matrix. PC1 and PC2 represent the two principal components, and the percentage indicates the contribution of the principal component to the sample difference. B. Permutational analysis of variance (PERMANOVA). The pseudo-F test showed statistically significant differences at $p < 0.05$, which are marked with *.

2.5 Analysis of the microbial diversity in the suppressive and non-suppressive soil samples

The structural diversity of bacterial communities at the phylum level is shown in Fig. 2. Actinobacteriota, Proteobacteria, Acidobacteriota, Chloroflexi, Firmicutes, and Gemmatimonadota were the six most abundant bacterial phyla and together accounted for more than 85% of all the observed ASVs. The contribution of the most abundant phyla to the studied bacterial populations of suppressive and non-suppressive soils was comparable in all the analysed soil samples.

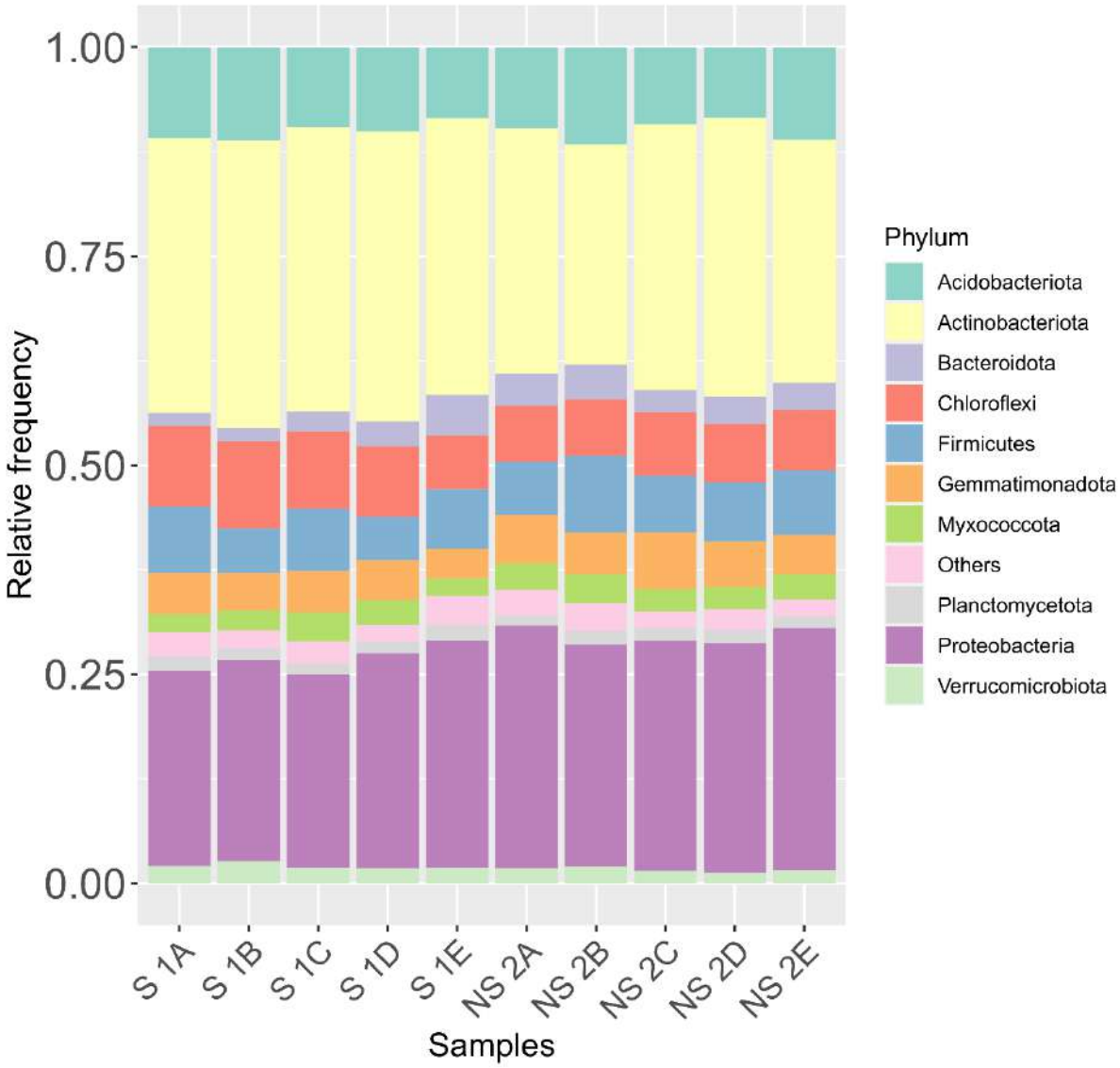

Figure 2. **Composition of the bacterial phyla in the suppressive (1A-1E) and non-suppressive soil samples (2A-2E)**, showing the relative frequency of the 10 most abundant phyla. S: suppressive soil, NS: non-suppressive soil

Our data are consistent with the analysis of the taxonomic composition of four potato farmlands in Italy presented by Cangioli et al. (2022) [37]. Four of the most numerous phyla in that study, *i.e.* Acidobacteriota, Actinobacteriota, Chloroflexi and Proteobacteria, were also among the most frequently detected taxa in the current study on Polish potato fields. A similar spectrum of the identified phyla was also observed in the sweet potato fields sampled from June to September in 2017 in three different locations in Japan [38].

Going down in the taxonomic rankings of the identified bacterial microbiota among suppressive and non-suppressive soil types (Fig. 3), a clear sharp clustering of the samples was visible, separating the suppressive soil from the non-suppressive soil, if the focus was turned into bacterial classes. The mostly abundant classes were Actinobacteria, Alphaproteobacteria, Gammaproteobacteria and Thermoleophilia. It is worth noting that among these highly abundant groups, the presence of

Thermoleophilia was shown to be higher in the suppressive soil in contrast to the non-suppressive soil ($p < 0.05$). On the contrary, concerning the other classes with low abundance, no significant differences based on the Wilcoxon rank sum test were found.

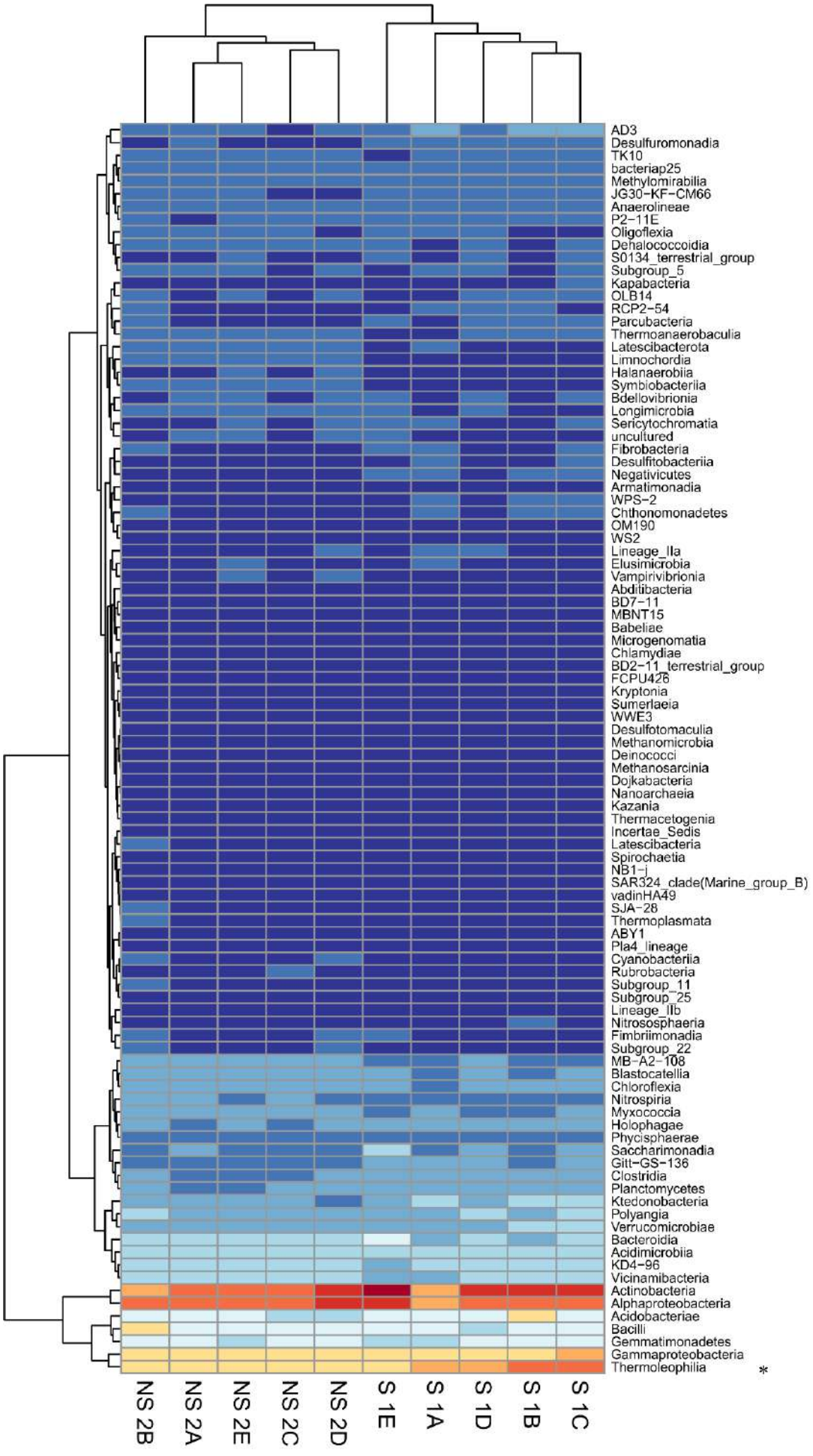
AD3
Desulfuromonadia
TK10
bacteriap25
Methylomirabilia
JG30−KF−CM66
Anaerolineae
P2−11E
Oligoflexia
Dehalococcoidia
S0134_terrestrial_group
Subgroup_5
Kapabacteria
OLB14
RCP2−54
Parcubacteria
Thermoanaerobaculia
Latescibacterota
Limnochordia
Halanaerobiia
Symbiobacteriia
Bdellovibrionia
Longimicrobia
Sericytochromatia
uncultured
Fibrobacteria
Desulfitobacteriia
Negativicutes
Armatimonadia
WPS−2
Chthonomonadetes
OM190
WS2
Lineage_IIa
Elusimicrobia
Vampirivibrionia
Abditibacteria
BD7−11
MBNT15
Babeliae
Microgenomatia
Chlamydiae
BD2−11_terrestrial_group
FCPU426
Kryptonia
Sumerlaeia
WWE3
Desulfotomaculia
Methanomicrobia
Deinococci
Methanosarcinia
Dojkabacteria
Nanoarchaeia
Kazania
Thermacetogenia
Incertae_Sedis
Latescibacteria
Spirochaetia
NB1−j
SAR324_clade(Marine_group_B)
vadinHA49
SJA−28
Thermoplasmata
ABY1
Pla4_lineage
Cyanobacteriia
Rubrobacteria
Subgroup_11
Subgroup_25
Lineage_IIb
Nitrososphaeria
Fimbriimonadia
Subgroup_22
MB−A2−108
Blastocatellia
Chloroflexia
Nitrospiria
Myxococcia
Holophagae
Phycisphaerae
Saccharimonadia
Gitt−GS−136
Clostridia
Planctomycetes
Ktedonobacteria
Polyangia
Verrucomicrobiae
Bacteroidia
Acidimicrobiia
KD4−96
Vicinamibacteria
Actinobacteria
Alphaproteobacteria
Acidobacteriae
Bacilli
Gemmatimonadetes
Gammaproteobacteria
Thermoleophilia
*
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
NS 2B
NS 2A
NS 2E
NS 2C
NS 2D
S 1E
S 1A
S 1D
S 1B
S 1C

Figure 3. **The clustered heatmap of ASVs identified in suppressive and non-suppressive soils according to the class level.** S: suppressive soil, NS: non-suppressive soil. If the Wilcoxon Rank Sum Test showed statistically significant differences at $p < 0.05$, they are marked with *.

When we moved to the order level, we noticed statistically significant differences between the investigated soil in relation to the Ktedonobacterales, Clostridiales, Saccharimonadales, Micropepsales, Frankiales, Acidobacteriales, Acetobacterales, Solirubrobacterales, Solibacterales, Chthoniobacterales, Streptomycetales and Xanthomonadales orders (Fig. 4). The Acidobacteriales, Acetobacterales, Streptomycetales and Bacillales seemed to be of a particular interest as strains of these orders have been described as efficient factors inhibiting the growth of pathogenic bacteria [17].

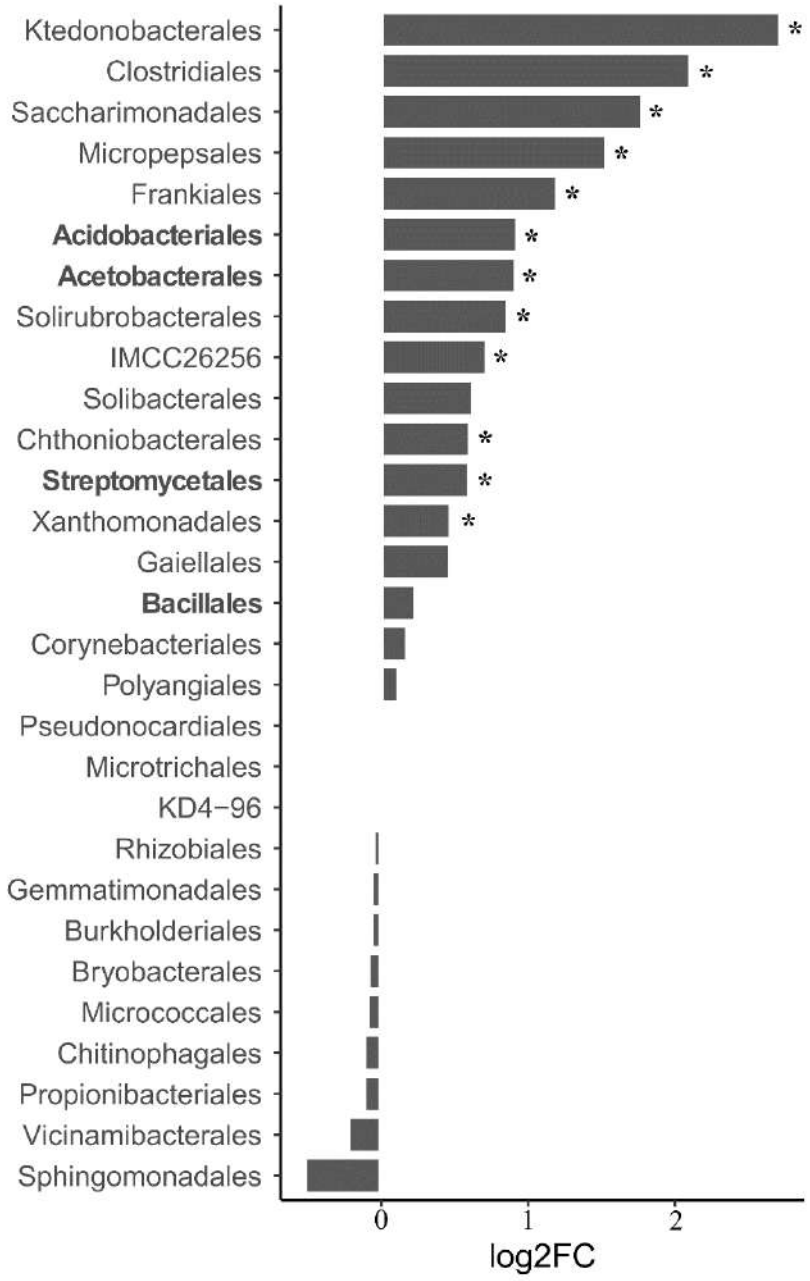

Figure 4. **The log2fold change among 30 taxa depicted at the order level among suppressive and non-suppressive soils**. Values above zero indicate that a stated order is more abundant in the non-suppressive soil, whereas log2fold changes below the zero point to the higher abundance of a certain

order in the suppressive soil. Taxa, which are according to the literature known for involvement in the control of plant pathogens, were displayed in bold [39]. If the Wilcoxon Rank Sum Test showed statistically significant differences at $p < 0.05$, they are marked with *.

The purpose of further analysis was to investigate in detail at the lower taxonomic level the differences in the abundance of members of the Acidobacteriales, Acetobacterales, Streptomycetales and Bacillales orders in soil suppression towards SRP. Finally, the comparison of the normalized counts for the four most divergent genera between the suppressive and non-suppressive soil samples was performed (Fig. 5). The most divergently abundant genera present in all the suppressive soil samples have been identified as *Bacillus*, *Rummeliibacillus*, *Acidobacterium* and *Gaiella*. The total number of occurrences of ASVs assigned to the genera *Bacillus* and *Rummeliibacillus* in the suppressive soil was 209 and 135, respectively, while in the non-suppressive soil they were not found at all. In turn, the number of ASVs corresponding to the *Gaiella* genus in the suppressive soil was 188 and *Acidobacterium* 133. As in the case of *Bacillus* and *Rummeliibacillus*, the corresponding ASVs did not occur in the non-suppressive soil samples. The obtained results may suggest that within the genus *Bacillus* and *Rummeliibacillus* there could be species that correspond to the suppressiveness of the analysed soil.

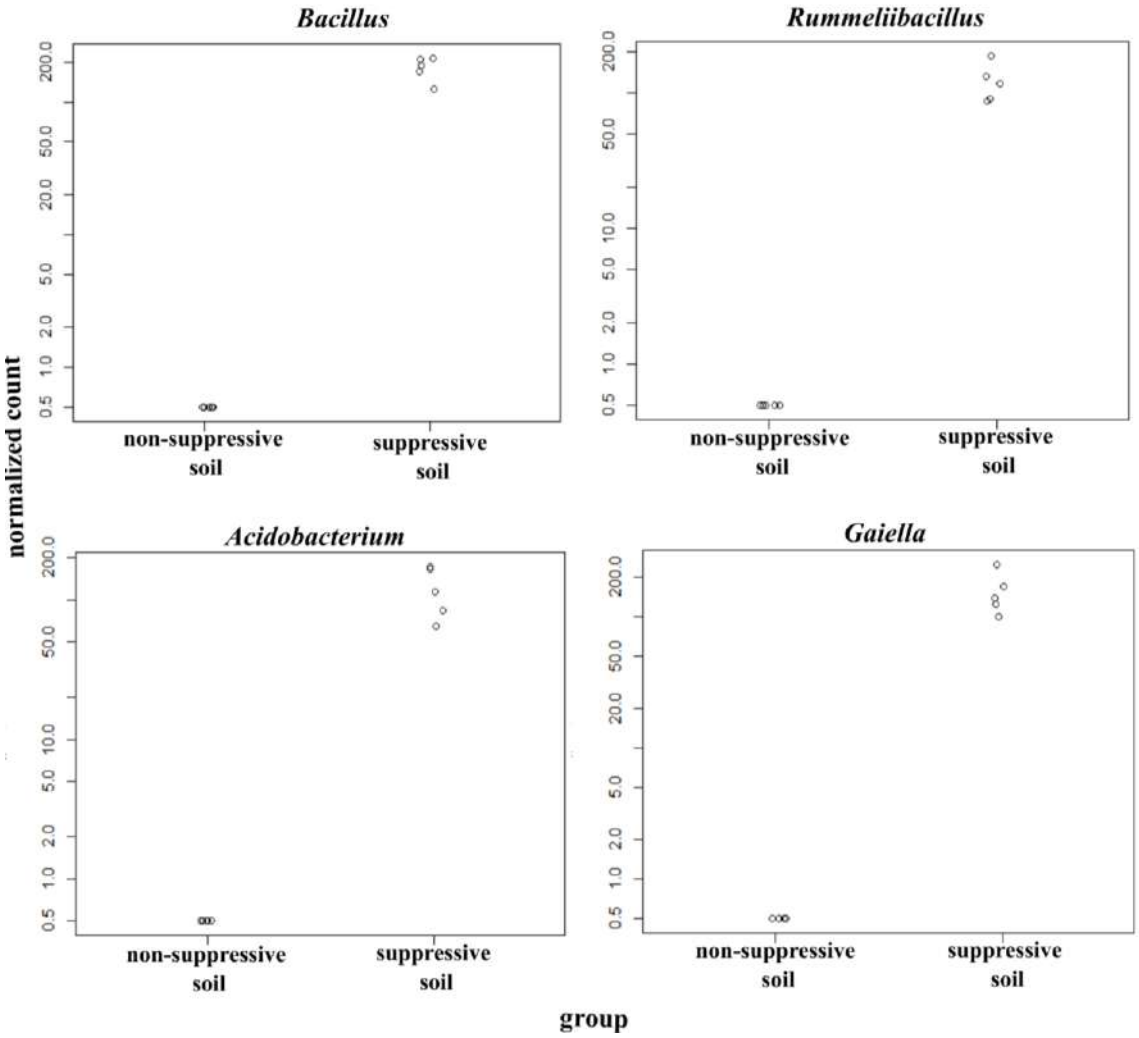

Figure 5. **Comparison of the normalized counts for the four most differently represented ASVs between the suppressive and non-suppressive soils towards SRP.**

Noteworthily, the representatives of the genus *Bacillus* were identified more frequently in the suppressive than the non-suppressive soil samples, which is in accordance with the fact that bacteria from the strain *Bacillus subtilis* DSM29784 are applied as plant growth promoting rhizobacteria (PGPR) [40]. The use of bioformulations with these microbes was stated to restore the biological balance and self-regulating capacity of the soil [39]. For example, in the search for biocontrol agents that could control soft rot diseases, *Bacillus amyloliquefaciens* subsp. *plantarum* [41] and *Myxococcus* spp. [42] isolated from soil have been used. Moreover, Weinert et al., (2010) observed, while studying microbial diversity on the surface of the soil-grown potato tubers, that the occurrence of *Bacillus* spp. and *Streptomyces* spp. was responsible for the inhibition of the development of disease symptoms caused by potato pathogens *Rhizoctonia solani, Verticillium dahliae* or *Phytophthora infestans* [43]. What could also be relevant is that bacteria from the *Bacillus* spp. can improve the fertilization efficacy and provide plants with elements from the limited soil resources, *e.g.* phosphorus, but also nitrogen, potassium, manganese and iron [44]. Additionally, bacteria from this genus exhibit direct or indirect beneficial effects towards plant growth *via* supporting the acquisition of nutrients, the production of phytohormones, antagonistic activity against phytopathogens and protection against abiotic stresses [45].

Former studies on soil suppressiveness in relation to the fungal root pathogen *Rhizoctonia solani* showed that subjection of soil to selective heat treatment at 80°C for 1 h eliminates the suppression of such soil. This observation was associated with a decrease in the numbers of three actinomycetes families, including *Streptomycetaceae* [20]. In turn, *Gaiella* spp. is one of the representatives of the Actinomycetia class, so far isolated from the aquatic and soil environment. Zhao et al. (2019) showed a strong positive relationship between the abundance of bacteria from the *Gaiella* group and soil chemistry (pH, NH_4^+, NO_3, soil organic matter and dissolved organic carbon) [46]. A strong linkage between the physicochemical properties of the soil and the presence of *Gaiella* spp. was also demonstrated during the analyses of soil in sugarcane fields [47]. Regarding Acidobacteria, the studies on soil suppressiveness in relation to vanilla *Fusarium* wilt disease conducted by Xiong et al. (2017) revealed a significant dominance of the bacterial phyla Acidobacteria, Actinobacteria and Firmicutes in the suppressive soil [48]. Among others, Acidobacteria participate in biogeochemical cycles, involving carbon, nitrogen or sulphur, by these means contributing to the balance in the plant-soil ecosystem. These microorganisms not only play a critical role in organic matter recycling by increasing the contents of organics and nitrogen in the soil along with boosting the availability of essential macroelements and microelements, but also acidobacteria secrete exopolysaccharides, which participate in the formation of the soil matrix and contribute to plant growth promotion by facilitating the water and nutrients uptake [49]. In addition, some species of acidobacteria produce a

phytohormone indole-3-acetic acid (IAA) and siderophores, exhibiting activities that significantly improve plant growth parameters [50].

3. Conclusions

The presented case study showed, for the first time, that the bacterial microbiota of the analysed suppressive and non-suppressive soil samples towards SRP revealed notable differences. In particular, the tested soils exhibited variations in the abundance of bacteria from the genera *Bacillus*, *Acidobacterium* and *Gaiella*, which were more frequently identified in the suppressive soil than the non-suppressive soil. The presented results suggest that differences in the composition of soil microbiota in potato fields of diverse soft rot and blackleg incidences might be related to soil suppressiveness. In our suggestion, it would be worth to examine the soil microbiota in order to predict blackleg and soft rot incidences on the fields and on this basis select the most suitable locations for the cultivation of elite seed potatoes. Moreover, this research might form a future perspective for the preparation of complex, effective biological formulations that may be applied as biocontrol agents for agricultural purposes.

4. Materials and Methods

4.1 Sampling of potato fields and physicochemical analysis of the soil

Soil samples designated as suppressive or non-suppressive towards soft rot and blackleg diseases were collected on July 2021 from potato fields located in Poland, the villages of Siemysl (54°38′ E, 18°58′ N, cultivar Lady Rosetta) and Bonin (54°15′ E, 16°24′ N, cultivar Denar), respectively. At the time of sampling and in the previous week, it was sunny and the air temperature approximated 30°C at 12.00 p.m. The distribution of soils and mineral formations of the collected soil samples were assigned on the basis of the soil classification system of the Polish Society of Soil Science classification of grain size. From each field, five soil samples were collected with a sterile metal spatula and placed directly into sterile plastic string bags. The soil was collected directly from beneath the potato plants at a depth of 20 cm. In each field, the sampling points were located at 5 m distance from each other. The soil samples intended for DNA isolation were immediately frozen and kept on dry ice. The samples designated for microbiological and chemical analyses were transported to the Laboratory of Plant Protection and Biotechnology at the Faculty of Biotechnology University of Gdansk and Medical University of Gdansk (IFB UG & MUG) at an ambient temperature and then stored one week at 4°C. In addition, soil samples and potato tubers were collected during harvest in August and sent to IFB UG & MUG for analyses involving the detection and identification of pectinolytic bacteria.

The chemical composition of the soil samples was determined by the District Chemical and Agricultural Station in Gdansk, Poland. From each field, three samples were analysed in terms of their pH, salinity and the contents of their microelements and macroelements such as: N-NO_3, P, K, Mg, Ca, Cl, Na, Zn, Cu, Mn, Fe, B, organic C and organic substance. The analysis was performed in accordance with the research procedure including the scope of accreditation for the testing laboratory for horticultural soil (No AB 787). Additionally, the moisture of the collected soil samples was

examined by a gravimetric method [51]. Then, the percentage average moisture content (weight; W) was calculated as the ratio of the water mass present in the soil (Mw) to the mass of the solid components of the soil post drying (mass after drying; Ms): $W = \frac{Mw}{Ms} x100\%$. In order to confirm the statistical significance of the obtained results, the t-student tests were performed.

4.2 Isolation and identification of *Dickeya* and *Pectobacterium* spp. strains by culture and molecular diagnostics approaches

The soil and potato tuber samples collected from two potato fields were tested for the presence of pectinolytic bacteria from the *Dickeya* spp. and *Pectobacterium* spp. using the method previously described by Potrykus et al. (2016) [14]. Briefly, 10 ml of 50 mM phosphate buffer pH 7.2 was added to 1 g of soil or plant material and mixed. The plant samples were manually homogenized with a Bioreba device (Bioreba, Switzerland). Also pectate enrichment medium (PEM) [52] was used to cultivate pectinolytic bacteria present in the soil. In terms of the latter approach, 10 g of a soil sample was suspended in 50 ml of PEM and incubated for 48 h at 28°C with 140 rpm shaking. Subsequently, filtering of the soil suspension through a sterile paper disc was conducted. Then, the selected dilutions of the resulting homogenate or filtrate were plated on a CVP media [53] and incubated for 48 h at 28°C. Bacterial colonies forming characteristic cavities on the CVP medium were further replated on CVP, and then TSA (the composition of peptone K, soy peptone, NaCl, dipotassium hydrogen phosphate, glucose and agar) until the axenic culture state was reached.

Subsequently, the cells of the collected pectinolytic strains were lysed by suspending one bacterial colony in 1 ml of distilled water and providing cold shock conditions for 1 h at -20°C. Afterwards, bacterial suspensions were heated for 10 min at 99°C prior to transferring 100 µl of the resultant lysate to 400 ml of sterile distilled water. Multiplex PCR was performed to identify strains to the species level as described previously [54].

4.3 Extraction of the total DNA and sequencing of the 16S rRNA library

Whole DNA from both suppressive and non-suppressive soil samples was isolated with the use of a commercially available NucleoSpin Soil Mini kit (Macherey-Nagel, Duren, Germany) following the manufacturer's guidelines. During the isolation procedure, 300-400 mg of soil and the SL1 lysis buffer were utilized. Purification of the isolated DNA was accomplished with NucleoSpin gDNA Clean-up Mini kits (Macherey-Nagel, Duren, Germany). The quality and concentration of the isolated DNA was assessed spectrophotometrically with NanoDrop ND-1000 (Thermo Fisher Scientific, Minneapolis, MN, USA). At least 3 µg of DNA ($OD_{260/280}$ 1.8-2.0) was sent to Genomed (Warsaw, Poland) for the preparation of libraries and sequencing of amplicons of the hypervariable V3-V4 region of the 16S rRNA gene. In more detail, the 341F (5'-CCT ACG GGN GGC WGC AG-3') and 785R (5'-GAC TAC HVG GGT ATC TAA TCC-3') primers were used to amplify the selected 16S rRNA region. The preparation of the library was based on performing PCR reactions with a KAPA HiFi HotStart ReadyMix, attaching dual indices and Illumina sequencing adapters using the Nextera XT Index Kit. PCR reaction was performed with a Q5 Hot Start High-Fidelity 2x Master Mix (New England Biolabs,

Ipswich, USA). The DNA was sequenced on the Illumina MiSeq instrument post utilization of the Illumina v3 kit (Illumina, San Diego, USA) in order to generate 300 bp paired-end reads. An automatic preliminary report was generated on the MiSeq sequencer using the MiSeq Reporter (MSR) v2.6 software (Illumina, San Diego, USA).

4.4 Processing of the raw data, clustering and taxonomic assignment of the reads

Initial quality control of the reads including investigation of the error profile of individual samples and generation of the dynamic parameters for quality control was conducted with the FIGARO tool [55] by Genomed (Warsaw, Poland). Subsequently, pre-processing of the data relied on the removal of the adapter sequences and rejection of the short reads (< 30 nt) with the Cutadapt tool [56]. Final quality assessment of the filtered data was performed with FASTQC [57].

Bioinformatic analysis allowing for taxonomic classification of the soil-inhabiting microbes basing on 16S rRNA reads was carried out with QIIME 2 [58]. The obtained reads were denoised using the DADA2 pipline [59] and unique sequences of biological origin, *i.e.* Amplicon Sequence Variants (ASVs) were assigned. In the next step, taxonomic classification of the ASVs was performed using a self-trained naive Bayes classifier that acquired data from the reference Silva 138 nr_v138 database [60]. The annotated ASVs count tables were processed using the 'Phyloseq' R package (RStudio version 2021.09.0) [61].

4.5 Downstream analysis of the 16S rRNA gene amplicon sequencing data

For alpha diversity analyses, Evenness (Pielou's index) and Shannon indices were calculated to measure the diversity. Beta diversity was analysed by a non-parametric, multivariate statistical test Permutation Based Analysis of Variance (PERMANOVA) in addition to Principal coordinates analysis (PCoA) based on Bray-Curtis distances. Finally, rarefaction curves were generated. In order to confirm the statistical significance of the obtained results the Kruskal-Wallis test ($p < 0.05$) for alpha-diversity and pseudo-F test ($p < 0.05$) for beta-diversity were performed.

Visualization of the results and statistical analyses were performed in R. In the case of the clustered heatmap of ASVs, the relative frequencies were normalized as follows 1/(abs(log10(aggregated_data_phylum+0.000001))). The Wilcoxon Rank Sum Test was used as a statistical test ($p < 0.05$). Differential abundance analysis was carried out using the R package DeSeq2 by juxtaposition with the reference sequences deposited in two databases, *i.e.* NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Sequence Match – RDP (http: // rdp. Cme. Msu .edu ›seqmatch) in order to identify the ASVs/taxa differing in a statistically significant manner between the studied soil types [62]. The relative frequencies were normalized as follows 1/(abs(log10(*relative frequency*+0.000001))), where 0.000001 represents a pseudo-count introduced here to avoid mathematical inconsistency.

Declaration of Competing Interest
The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability
The 16S rRNA raw sequencing data are publicly available in the Sequence Read Archive repository at https://www.ncbi.nlm.nih.gov/sra/PRJNA902273. All data generated or analysed during this study are included in this published article (or in its supplementary information files).

Author Contributions
E. L., A. M-P and W. B-W. conceived and designed all experiments. W. B-W. collected soil samples, performed all microbiological and molecular diagnostics experiments, isolated total DNA from all the collected soil samples and performed bioinformatic analysis. W. B-W, M. F., A. M., A. E. M. took part in the discussion on the selection of appropriate bioinformatics tools for visualization of 16S rDNA sequencing data. W. B-W., M. F. visualized the collected data. W. B-W, A. M-P., A. M., E. L. took part in the discussion on the collected data. W. B-W. wrote the first version of this manuscript. W. B-W, A. M-P., A. M., E. L. prepared the final version of this manuscript that has been accepted by all the other authors. W. B-W. and E. L. acquired funding for these studies.

Competing interests
The authors declare no competing interests.

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Supplementary information

Differences in the constituents of bacterial microbiota in relation to soil suppressiveness towards potato blackleg and soft rot diseases

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Table S1: Raw data obtained from 16S rRNA gene amplicon sequencing analysis

Soil	No. of soil sample	Number of raw read pairs	% passing QC	number of unique ASVs
Suppressive soil	1A	95 493	78.14%	786
	1B	106 426	78.38%	922
	1C	117 623	75.36%	1007
	1D	111 729	77.05%	960
	1E	110 913	75.63%	941
Non-suppressive soil	2A	97 970	75.77%	803
	2B	111 036	75.84%	1024
	2C	911 24	74.33%	767
	2D	104 487	74.84%	924
	2E	101 293	74.92%	911

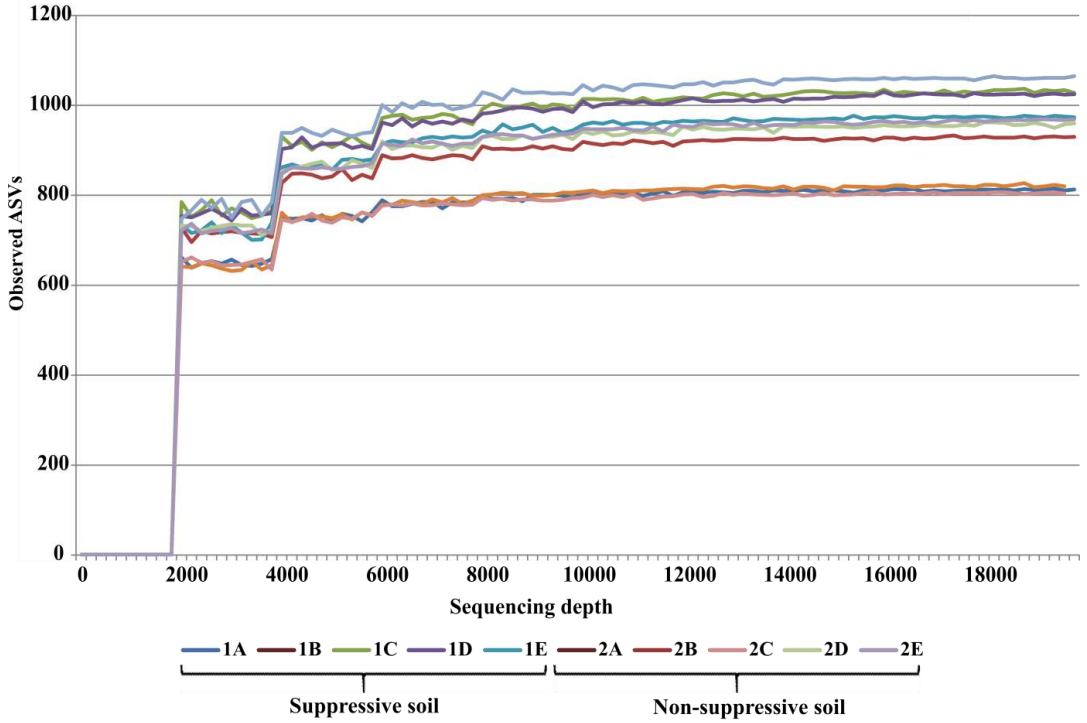

Figure S1. Rarefaction curve for suppressive and non-suppressive soil samples. The x axis indicates the sequencing depth, while the y axis corresponds to the number of the observed ASVs.

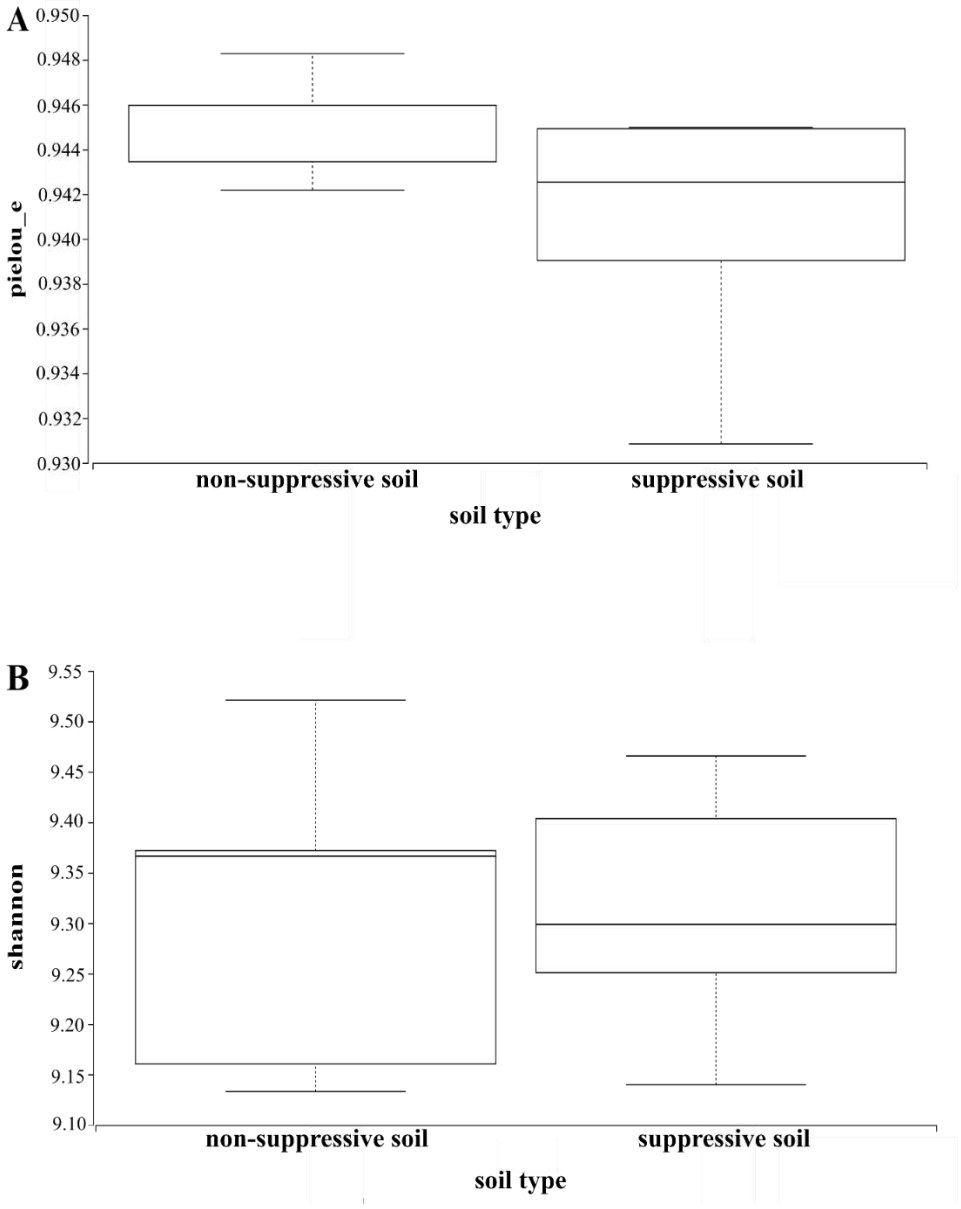

Figure S2. Alpha-diversity indices calculated for the studied suppressive and non-suppressive soil samples. A. Pielou's indices. B. Shannon index. Differences were not statistically significant based on Kruskal-Wallis tests ($p > 0.05$).

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AUTHOR CONTRIBUTION STATEMENT

I declare that in the manuscript:

"Differences in the constituents of bacterial microbiota in relation to soil suppressiveness towards potato blackleg and soft rot diseases"

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my contribution involved conceived and designed all experiments. Moreover I collected soil samples, performed all microbiological and molecular diagnostics experiments, isolated total DNA from all the collected soil samples, performed bioinformatic analysis and visualized the collected data. I took part in the discussion on the collected data. I prepared first version of this manuscript and corrected it according to the co-authors' suggestions.

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AUTHOR CONTRIBUTION STATEMENT

I declare that in the manuscript:

"Differences in the constituents of bacterial microbiota in relation to soil suppressiveness towards potato blackleg and soft rot diseases"

Weronika Babińska-Wensierska, Agata Motyka-Pomagruk, Marco Fondi, Agnieszka Emilia Misztak, Alessio Mengoni, Ewa Łojkowska

my involvement included participation in planning of the described experiments, corrections of the first version of the manuscript and discussion of the research results.

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my contribution involved in the in the discussion on the collected data and visualized the bioinformatic data. Moreover, I took part in the discussion on the collected data and provided correction to the first version of the manuscript.

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AUTHOR CONTRIBUTION STATEMENT

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my contribution took part in the discussion on the selection of appropriate bioinformatics tools for visualization of 16S rDNA sequencing data. Moreover, I provided correction to the first version of the manuscript.

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I declare that, I was responsible for co-conceptualization of the project and designed experiments. I participated in the discussion of the results and preparation of the final version of the manuscript.

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Dickeya paradisiaca comb. nov. and delineation of four novel species, *Dickeya dadantii* sp. nov., *Dickeya dianthicola* sp. nov., *Dickeya dieffenbachiae* sp. nov. and *Dickeya zeae* sp. nov. Int. J. Syst. Evol. Microbiol. 55, 1415–1427. https://doi.org/10.1099/IJS.0.02791-0
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6. *Curriculum vitae*

Personal data

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Education

2019 - present Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk

Intercollegiate Biotechnology Doctoral School of University of Gdańsk and Medical University of Gdańsk

2017 - 2019 Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk

Graduate studies in Biotechnology. Master's thesis "Antibacterial activity of post-plasma solutions generated by direct current atmospheric pressure glow discharge against bacterial phytopathogens"

2014 - 2017 Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk

Undergraduate studies in Biotechnology. Bachelor's thesis "Irrigation water sources as contributors to the spread of *Dickeya* and *Pectobacterium* spp. *via* soil, air and water local microenvironments"

2010 - 2013 1st High School in Gdańsk

Internships and Workplaces

10.2019 - present Employee at **Research and Development Laboratory** of the Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, accredited laboratory working in accordance with the quality management system ISO 17025 and environmental management system ISO 14001, Gdańsk, Poland

17-28.10.2022 Internship in **Research Institute of Pomology and Floriculture,**

	Department of Plant Pathology, under the guidance of Prof. Joanna Puławska, Skierniewice, Poland
22-24.08.2022 **14-18.12.2017** **01-03.02.2017**	Internship at **Wroclaw University of Science and Technology**, Department of Analytical Chemistry and Chemical Metallurgy, under the guidance of Prof. Piotr Jamróz, Wroclław, Poland
04.10.2021-10.01.2022	Internship at **University of Florence**, Dipartimento di Biologia - Laboratorio di Genetica Microbica, under the guidance of Prof. Alessio Mengoni, Florence
1.04-1.08.2018	Internship at **Laboratory of Plant Protection and Biotechnology**, Intercollegiate Faculty of Biotechnology University of Gdańsk and Medical University of Gdańsk, under the guidance of Prof. Ewa Łojkowska, Gdańsk, Poland
1.04-1.08.2018	Student exchange under the ERASMUS+ Programme at **Heinrich-Heine-Universität Düsseldorf**, Düsseldorf, Germany
06.2016	Internship in **Lipopharm.pl company**, Gdańsk, Poland

Publications & Book chapters

1. Śledź, W., Motyka-Pomagruk, A., Żukowska, D., **Babińska-Wensierska, W.,** Żolędowska, S., Łojkowska E. (2023). Genotypic and phenotypic uniformity among the population of *Pectobacterium atrosepticum* strains isolated during three growing seasons from potato fields in Poland. ***European Journal of Plant Pathology***. 167, 99–121. https://doi.org/10.1007/s10658-023-02687-y
2. Kowalczyk, A., **Babińska-Wensierska, W.,** Łojkowska, E., Kaczyński, Z. (2023). The structure of O-polysaccharide isolated from the type strain of *Pectobacterium versatile* CFBP6051T containing an erwiniose - higher branched monosaccharide. ***Carbohydrate Research.*** 524, 108743. https://doi.org/10.1016/j.carres.2023.108743
3. Terefinko, D., Caban, M., Motyka-Pomagruk, A., **Babińska, W.,** Pohl, P., Jamróz, P., Cyganowski, P., Śledź, W., Łojkowska, E., Stepnowski, P., Dzimitrowicz, A. (2023). Removal of clinically significant antibiotics from aqueous solutions by applying unique high-throughput continuous-flow plasma pencil and plasma brush systems. ***Chemical Engineering Journal,*** 452, 139415. https://doi.org/10.1016/j.cej.2022.139415
4. Dzimitrowicz, A., Caban, M., Terefinko, D., Pohl, P., Jamróz, P., **Babińska, W.,** Cyganowski, P., Stepnowski, P., Łojkowska, E., Śledź, W., Motyka-Pomagruk, A. (2022). Application of pulse-modulated radio-frequency atmospheric pressure glow discharge for degradation of doxycycline

from a flowing liquid solution. ***Scientific Reports***, 12(1), 1-16. https://doi.org/10.1038/s41598-022-11088-w

5. Kowalczyk, A., Szpakowska, N., **Babińska, W.,** Motyka-Pomagruk, A., Śledź, W., Łojkowska, E., Kaczyński, Z. (2022). The structure of an abequose-containing O-polysaccharide isolated from *Pectobacterium aquaticum* IFB5637. ***Carbohydrate Research***, 522, 108696. https://doi.org/10.1016/j.carres.2022.108696
6. **Babińska, W.,** Motyka-Pomagruk, A., Śledź, W., Kowalczyk, A., Kaczyński, Z., Lojkowska, E (2021). The First Polish Isolate of a Novel Species *Pectobacterium aquaticum* Originates from a Pomeranian Lake. ***International Journal of Environmental Research and Public Health***, 18(9), 5041. http://dx.doi.org/10.3390/ijerph18095041
7. Motyka-Pomagruk, A., Dzimitrowicz, A., Orłowski, J., **Babińska, W.,** Terefinko, D., Rychłowski, M., Prusiński, M., Pohl, P., Łojkowska, E., Jamróz, P., Śledź, W. (2021). Implementation of a NonThermal Atmospheric Pressure Plasma for Eradication of Plant Pathogens from a Surface of Economically Important Seeds. ***International Journal of Molecular Sciences***, 22, 9256. http://dx.doi.org/10.3390/ijms22179256
8. Dzimitrowicz, A., Jamróz, P., Pohl, P., **Babińska, W.,** Terefinko, D., Śledź, W., Motyka-Pomagruk, A. (2021). Multivariate Optimization of the FLC-dc-APGD-Based Reaction-Discharge System for Continuous Production of a Plasma-Activated Liquid of Defined Physicochemical and AntiPhytopathogenic Properties. ***International Journal of Molecular Sciences***, 22(9), 4813. http://dx.doi.org/10.3390/ijms22094813
9. Dzimitrowicz, A., Motyka, A., Jamróz, P., Łojkowska, E., **Babińska, W**., Terefinko, D., Pohl, P., Śledź, W. (2018). Application of silver nanostructures synthesized by cold atmospheric pressure plasma for inactivation of bacterial phytopathogens from the genera *Dickeya* and *Pectobacterium*. ***Materials*** 11(3), 331. https://doi.org/10.3390/ma11030331
10. Dzimitrowicz, A., Motyka-Pomagruk, A., Cyganowski, P., **Babińska, W**., Terefinko, D., Jamróz, P., Łojkowska, E., Pohl, P., Śledź, W. (2018). Antibacterial activity of fructose-stabilized silver nanoparticles produced by direct current atmospheric pressure glow discharge towards quarantine pests. ***Nanomaterials*** 8(10), 751. http://doi.org/10.3390/nano8100751
11. Dzimitrowicz, A., Motyka, A., Śledź, W., Jamróz, P., Łojkowska, E., **Babińska, W**., Pohl, P. (2017). Eradykacja bakteryjnych patogenów roślin przy zastosowaniu wyładowań jarzeniowych generowanych pod ciśnieniem atmosferycznym (ang. Eradication of bacterial plant pathogens using glow discharges generated at atmospheric pressure). ***Nauka i przemysł-lubelskie spotkania studenckie, Book chapter.*** ISBN 9788393946594

Submitted manuscript:

12. **Babińska-Wensierska, W.**, Motyka-Pomagruk, A., Fondi, M., Misztak, A. E., Mengoni, A., Łojkowska, E. (2023). Differences in the constituents of bacterial microbiota in relation to soil suppressiveness towards potato blackleg and soft rot diseases. Submitted for publication

13. **Babińska-Wensierska, W**., Motyka-Pomagruk, A., Mengoni, A., DiCenzo, G., Łojkowska, E. (2023). Search for foundations of virulence in an economically significant plant pathogen *Dickeya solani* via transcriptomic profiling. Submitted for publication
14. Agata Motyka-Pomagruk, **Weronika Babinska-Wensierska**, Wojciech Sledz, Anna-Karina Kaczorowska, Ewa Lojkowska (2023). Phyloproteomic study by MALDI-TOF MS in view of intraspecies variation in a significant homogenous phytopathogen *Dickeya solani*. Submitted for publication

Obtained patents

1. „Method of producing a plant growth stimulation preparation, preparation obtained by this method and use of the preparation to stimulate the growth of plants, in particular those of economic importance". Polish patent Pat. 236377. 2020-03-17. Motyka-Pomagruk Agata, Dzimitrowicz Anna, **Babińska Weronika**, Śledź Wojciech, Pohl Paweł, Jamróz Piotr, Terefinko Dominik, Łojkowska
2. "Method for eradication of bacterial phytopathogens". Polish patent, Pat. 236665. 2020-10-13. Dzimitrowicz Anna, Motyka-Pomagruk Agata, Jamróz Piotr, Śledź Wojciech, **Babińska Weronika**, Pohl Paweł, Łojkowska Ewa

Patent applications

1. PCT International Patent Application. „Method of degrading antibiotics from aqueous solutions by using cold atmospheric pressure plasma generated in a flowing plasma brush and a plasma brush intended for this method". PCT/PL2022/050027. 06.05.2022. Dzimitrowicz Anna, Motyka-Pomagruk Agata, Caban Magda, **Babińska Weronika**, Terefinko Dominik, Śledź Wojciech, Jamróz Piotr, Cyganowski Piotr, Łojkowska Ewa, Stepnowski Piotr, Pohl Paweł
2. EPO international patent application. „Method for eradication of pathogenic microorganisms from flat surfaces or skin tissue and a system for carrying out this method". EP23169144.5. 21.04.2023. Motyka-Pomagruk Agata, Śledź Wojciech, Prusiński Michał, Orłowski Jakub, **Babińska Weronika**, Łojkowska Ewa, Dzimitrowicz Anna, Jamróz Piotr, Pohl Paweł, Jerzy Dora
3. Polish patent application „Sposób eradykacji drobnoustrojów chorobotwórczych z powierzchni płaskich lub tkanki skórnej oraz układ do realizacji tego sposobu" (ang. „A method of eradicating pathogenic microorganisms from surfaces planar or skin tissue and a system for carrying out this method"). P.441356. 02.06.2022. Motyka-Pomagruk Agata, Śledź Wojciech, Prusiński Michał, Orłowski Jakub, **Babińska Weronika**, Łojkowska Ewa, Dzimitrowicz Anna, Jamróz Piotr, Pohl Paweł
4. Polish patent application „Sposób ochrony roślin istotnych gospodarczo, zwłaszcza roślin naczyniowych, przed patogenami bakteryjnymi" (ang. „A method of protecting economically important plants, especially vascular plants, against bacterial pathogens"). P.438360. 06.07.2021. Dzimitrowicz Anna, Jamróz Piotr, Pohl Paweł, Terefinko Dominik, Motyka-Pomagruk Agata, **Babińska Weronika**, Śledź Wojciech, Łojkowska Ewa, Orłowski Jakub, Prusiński Michał

5. Polish patent application „Sposób degradacji antybiotyków z roztworów wodnych z zastosowaniem nietermicznej plazmy atmosferycznej" (ang. „Method of degradation of antibiotics from aqueous solutions using non-thermal atmospheric plasma"). P.437603. 17.04.2021. Dzimitrowicz Anna, Śledź Wojciech, Caban Magda, Jamróz Piotr, Motyka-Pomagruk Agata, **Babińska Weronika**, Cyganowski Piotr, Terefinko Dominik, Stepnowski Piotr, Pohl Paweł, Łojkowska Ewa
6. Polish patent application „Sposób rozkładu antybiotyków z roztworów wodnych z zastosowaniem zimnej plazmy atmosferycznej, generowanej w przepływowej szczotce plazmowej oraz szczotka plazmowa do realizacji tego sposobu" (ang. „Method of decomposing antibiotics from aqueous solutions using cold atmospheric plasma generated in a flow plasma brush and a plasma brush for implementing this method"). P.440185. 10.01.2022. Dzimitrowicz Anna, Motyka-Pomagruk Agata, Caban Magda, **Babińska Weronika**, Terefinko Dominik, Śledź Wojciech, Jamróz Piotr, Cyganowski Piotr, Łojkowska Ewa, Stepnowski Piotr, Pohl Paweł

Research projects

Principal investigator:

Preludium 21 "Does the microbiome of arable soil influence development of blackleg and soft rot diseases caused by pectinolytic bacteria of the *Dickeya* and *Pectobacterium* genera?", 2022/45/N/NZ9/01923, granted to M.Sc. Weronika Babińska-Wensierska by the National Science Centre in Poland financed by National Science Centre in Poland

Project executor:

Harmonia 6 "The application of pangenome-based approach to identify genes responsible for adaptation of pectinolytic bacteria - *Dickeya solani* and *Pectobacterium wasabiae* to causing disease symptoms on potato under temperate climate conditions", 2014/14/M/NZ8/00501, granted to Prof. Ewa Łojkowska by the National Science Centre in Poland

Preludium 11 "Gene expression dynamics of main *Dickeya solani* virulence factors during the infection process in planta", UMO-2016/21/N/NZ1/02783, granted to Ph.D. Agata Motyka-Pomagruk by the National Science Centre in Poland

Innovation Incubator 2.0 "Method of preparing plant material and the method detection and identification of bacteria of the species *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium atrosepticum* and bacteria of the genus *Dickeya* spp.", MNiSW/2019/169/DIR, financed by the EU Operational Program Intelligent Development 2014-2020, granted to Prof. Ewa Łojkowska

Opus 17 "Assessment of the antibacterial properties of post-plasma solutions generated with the use of cold atmospheric pressure plasmas against economically important phytopathogens and impact of the obtained liquids on the growth of crops and vegetables", 2018/31/B/NZ9/03685, granted to Ph.D. Eng. Wojciech Śledź by the National Science Centre in Poland

Sonata 15 "Application of cold atmospheric pressure plasmas generated in contact with a flowing liquid for direct degradation of antibiotics and limitation of multi-drug resistance in the natural

environment", 2019/35/D/ST8/04107, granted to Ph.D. Eng. Anna Dzimitrowicz, Professor PWr/ Ph.D. Eng. Wojciech Śledź by the National Science Centre in Poland

Innovation Incubator 4.0 "Integration of documentation and testing procedure for the detection of *Pectobacteriaceae* in plant material with the ISO 14001:2015 system" , 7899- 2:2000, MNiSW/2020/317/DIR granted to Prof. Ewa Łojkowska

Innovation Incubator 4.0 "Extending the scope of laboratory accreditation research and implementation of MWB UG for microbiological analyzes of water in accordance with ISO 6222:2004and 9308-1:2014", MNiSW/2020/317/DIR, granted to Ph.D. Eng Wojciech Śledź

Opus 23 "Development of new processes for the production and sterilization of functional plant drinks with the use of cold atmospheric plasma technology", 557-T014-0285-23-2A, granted to Ph.D. Eng. Anna Dzimitrowicz, Professor PWr by the National Science Centre in Poland

Participation in international research cooperation under the following projects:

Global Initiative of Crop Microbiome and Sustainable Agriculture. Head: Brajesh K Singh

Fields4ever (947084) UE Horizon 2020 „A global soil health initiative". Head: Javier Bobo-Pinilla

Scholarships and awards

Scholarship of the Minister of Science and Higher Education for outstanding scientific achievements for the academic year 2018/2019 decision no. DBF.WPM.633.96.2018.GW

Rector's Scholarship for the best students at the University of Gdansk in academic years 2014/2015, 2016/2017, 2017/2018, 2018/2019

Marshal's of the Pomeranian Voivodeship Scholarship for the best students for the academic year 2017/2018

A special award from the President of the Patent Office of the Republic of Poland (2020) in the 10th edition of the National Student-Inventor Competition for the solution „Method of producing a plant growth stimulation preparation, preparation obtained by this method and use of the preparation to stimulate the growth of plants, in particular those of economic importance". polish patent, Pat. 236377

Honored in the DGP EUREKA - Discovering Polish Inventions competition (2020) for the invention titled "Method for eradication of bacterial phytopathogens" Anna Dzimitrowicz, Agata Motyka-Pomagruk, Piotr Jamróz, Wojciech Śledź, **Weronika Babińska**, Ewa Łojkowska, Paweł Pohl

Rector's 2nd Degree Award for scientific achievements in the field of exact and natural sciences in the academic year 2021/2022

1st place in the 2nd edition of the **Young Fahrenheit** competition (2023) for the pro-ecological deactivation method phytopathogens and stimulation of the growth of plants with high commercialization potential Agata Motyka-Pomagruk, **Weronika Babińska,** Wojciech Śledź

Courses and training

Research and analytical competences:

- "Operating autoclaves and sterilizers" - Professional Development Department, Gdańsk, Poland, 30.09.2017
- „Methods in molecular biology – laboratories" Gdańsk, Poland, 5-9.06.2017
- „Synthetic biology: New concepts and methods" Gdańsk, Poland, 30.09.2019-04.10.2019
- „Preparation of libraries for RNA-Seq" Warszawa, Poland, 29-30.11.2019
- „Introduction to RNA-Seq data analysis" Poznań, Poland, 14-15.12.2019
- "Programmer Specialty: Python Basics" Warszawa, Poland, 18.04.2020
- " Introduction to metagenomics" Poznań, Poland, 26-27.06.2021
- Completed online courses on the Datacamp e-learning platform: „Introduction to R" (completed on 14.10.2021) and „Intermediate R" (completed on 18.10.2021)
- "Microbiome & Health" e-learning platform, 25.07.2022
- „Analysis of Prokaryotic RNA-Seq data", Gdańsk, Poland, 10-11.12.2022
- „High Performance Liquid Chromatography UPLC/HPLC in practice", Gdańsk, Poland, 05-07.10.2023
- „Analysis and visualization of biological data in R" Poznań, Poland, 17-20.04.2023

Quality assurance, management systems and ISO standards requirements:

- "Requirements of the new standard PN-EN ISO/IEC 17025:2018-02" Gdańsk, Poland 29-30.10.2019
- „Requirements of the standard PN-EN ISO 14001: 2015-09 Environmental management systems. Requirements and guidelines for use" Gdańsk, Poland 19.11.2019
- „Internal audit of the Environmental Management System in accordance with the requirements of the ISO 14001:2015 standard" Gdańsk, Poland 23.02.2022

Language

- English: advanced level (C1 certificate)
- German: basic level
- French: basic level

Conference reports (44)

1. **Babińska-Wensierska W**., Smorawiński K., Motyka-Pomagruk A., Śledź W., Łojkwoska E. "Biodiversity of bacterial plant pathogens from the *Pectobacteriaceae* family on Polish waterways" 12th International Congress of Plant Pathology, Lyon, France, 20-25.08.2023, Poster presentation
2. Motyka-Pomagruk A., Dzimitrowicz A., **Babińska-Wensierska W**., Orłowski J., Terefinko D., Prusiński M., Jamróz P., Pohl P., Łojkowska E., Śledź W. „Perspectives on the application of cold

atmospheric pressure plasmas in the plant protection filed" 12th International Congress of Plant Pathology, Lyon, France, 20-25.08.2023, Co-author of poster presentation

3. Śledź W., Motyka-Pomagruk A., **Babińska-Wensierska W**., Żukowska D., Żolędowska S., Łojkowska E. "Genotypic and phenotypic analyses on *Pectobacterium atrosepticum* associated with potato soft rot and blackleg diseases in Poland" *Pectobacteriaceae* satellite event, Lyon, France, 19-20.08.2023. Co-author of oral presentation
4. **Babińska-Wensierska W**., Motyka-Pomagruk A., Fondi M., Mengoni A., Łojkowska E. "Does the microbiome of soil influence development of blackleg and soft rot diseases caused by *Pectobacteriaceae*?" *Pectobacteriaceae* satellite event, Lyon, France, 19-20.08.2023. Oral presentation
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