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DOCTORAL THESIS

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Synthetic consortium of bacterial strains protecting potato (*Solanum tuberosum* L.) plants against pectinolytic bacteria: form Petri dish to the potato storage

Syntetyczne konsorcjum bakterii chroniące rośliny ziemniaka (*Solanum tuberosum* L.) przed bakteriami pektynolitycznymi: od szalki Petriego do przechowalnictwa ziemniaków

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Abstract

Bacterial diseases of plants cause considerable losses in agriculture due to yield damages and deterioration of the quality of plant produce. Worldwide, agriculture relies primarily on chemical control methods (e.g. pesticides) and prevention to protect crops from pathogens. However, extensive usage of such substances in farming leads to severe ecological problems. Furthermore, it is postulated that the growing resistance of plant pathogens to chemicals used in agricultural applications will increase disease incidence in the near future. An excellent example of such a disease is potato soft rot caused by bacteria belonging to Soft Rot *Pectobacteriaceae* (SRP: *Pectobacterium* spp. and *Dickeya* spp.). Until now, control of potato soft rot relies mainly on prevention, good agricultural practices, and pathogen-free seed material usage.

Biological plant protection is an ecologically friendly alternative to chemical control methods to protect crops from diseases. This method utilizes the natural ability of microorganisms to limit the growth of each other. For example, beneficial bacteria, which produce a wide array of antimicrobial compounds, can be used to prevent the spread of pathogens. However, even though this method seems promising, its application encounters several technical difficulties.

Firstly, most of the research on using microorganisms for such purposes finishes before any bioactive product is developed and introduced to the market. Likewise, the selected beneficial strains are not usually broadly evaluated under natural conditions under which the crop is maintained. Therefore, even though microorganisms may have promising antimicrobial activity under laboratory conditions, the obtained results cannot be translated directly into an economic profit and marketed products. Additionally, most of the research presented so far employs only single strains of microorganisms, not their combinations or mixtures.

The reason is that the products containing only one type of microorganism are easier to register for marketing. However, they have a narrower activity range than products containing several beneficial strains combined in one product and supporting each other.

My doctoral dissertation aimed to develop an artificial (synthetic) consortium of bacterial strains effective against SRP bacteria. Furthermore, I developed a formulation to improve the shelflife of the consortium and subsequently tested the formulated mixture containing bacterial strains for the protective activity against SRP bacteria in potato tubers under real-life storage conditions.

During the experiments on potato tubers, the consortium of five strains of Gram-negative bacteria: *Serratia plymuthica* A294, *Lellitottia amnigena* A167, *Rahnella aquatilis* H145, *Serratia rubidaea* H440 and H469 was developed, based on the disease suppressing activity. Subsequently, I tested the preservation methods for increasing the survival of synthetic consortium during storage using wettable powder formulations. Afterwards, I evaluated the prepared formulations in 6-month experiments on potato tubers kept under storage conditions mimicking natural conditions used to store commercial potato tubers. Finally, I assessed the interactions of the selected bacterial strains and their antagonisms.

The obtained results led to the development of an artificial (synthetic) consortium with the proposed formulation, which could be applied on seed tubers under storage for protection against potato soft rot. The obtained results were published in three experimental research publications.

The first article describes the development and evaluation of the artificial (synthetic) consortium of microorganisms. The second publication deals with the design, development and evaluation of the formulation of the synthetic consortium.

Finally, the last publication describes the genomes of the strains comprising artificial (synthetic) consortium and genome-based characteristics of their biological control activity.

The practical aspect of my PhD project has led to three patents (two Polish and one European patent) describing: (i) the usage of the designed synthetic consortium, (ii) the way to formulate the consortium to prolong its shelf life and (iii) a lyophilization reagent that was developed during my studies.

In summary, my research led to the development and evaluation of an innovative artificial (synthetic) consortium containing five bacterial strains that can be used to protect potato tubers against *Pectobacterium* spp. and *Dickeya* spp. under storage conditions. In a series of experiments, this consortium was evaluated for its activity and preparation stability to be introduced on the market. In addition, the members of the artificial (synthetic) consortium were tested to see whether they could negatively influence each other and whether the consortium's activity was based on the cooperative action of its members.

Such analysis of the microbial consortia can help us understand the complexity of interactions between antagonistic bacteria both in artificial and in natural settings.

Streszczenie

Bakteryjne choroby roślin powodują istotne straty w rolnictwie ze względu na utratę plonów oraz obniżenie jakości produktów rolnych. Obecnie w celu ochrony roślin przed patogenami, światowa produkcja rolnicza polega głównie na zastosowaniu środków chemicznych (pestycydów), jednakże używanie tych substancji w dużych ilościach może prowadzić do poważnych problemów ekologicznych. Podejrzewa się, że zwiększanie oporności patogenów roślin na chemiczne środki ochrony rośli spowoduje zwiększenie częstotliwości występowania tych chorób w przyszłości. Doskonałym przykładem takiej choroby jest mokra zgnilizna ziemniaków powodowana przez bakterie pektynolityczne należące do grupy ang. Soft Rot *Pectobacteriaceae* (SRP: *Pectobacterium* spp. i *Dickeya* spp.). Do tej pory walka z mokrą zgnilizną polegała głównie na zapobieganiu zakażeniom, dobrych praktykach rolniczych i korzystaniu z materiału siewnego wolnego od patogenów.

Biologiczna ochrona roślin to przyjazna dla środowiska alternatywa w stosunku do metod chemicznych. Metoda ta wykorzystuje naturalną zdolność mikroorganizmów do wzajemnego hamowania wzrostu. Na przykład, pożyteczne bakterie, które produkują szerokie spektrum substancji antybiotycznych mogą być wykorzystane, aby zapobiec rozprzestrzenianiu się patogenów. Pomimo że jest to obiecująca metoda, jej zastosowanie jest ograniczone z uwagi na przeszkody techniczne.

Po pierwsze, większość badań z wykorzystaniem mikroorganizmów do takich celów kończy się zanim produkt zostanie opracowany i wprowadzony na rynek. Następnie, wybrane szczepy bakteryjne, zwykle nie są szczegółowo badane w warunkach polowych. W związku z tym, mimo zachęcających wyników badań wstępnych przeprowadzonych w warunkach laboratoryjnych, mogą one nie znaleźć prostego przełożenia na zysk ekonomiczny i działanie produktu powstałego z zastosowaniem tych mikroorganizmów.

Dodatkowo, większość prac eksperymentalnych przeprowadzana jest na pojedynczych szczepach, a nie mieszaninach różnych szczepów mikroorganizmów. Powodem tego stanu rzeczy, jest fakt, że produkty powstałe na bazie jednego typu mikroorganizmu są łatwiejsze w rejestracji i komercjalizacji. Jednakże takie produkty mają jednocześnie węższe spektrum działania niż te zawierające wiele szczepów mikroorganizmów w jednym preparacie.

Moja praca doktorska miała na celu opracowanie syntetycznego konsorcjum szczepów bakterii antagonistycznych względem bakterii SRP, opracowanie formulacji w celu zwiększenia stabilności półkowej mieszaniny, a następnie przetestowanie formulacji zawierającej syntetyczne konsorcjum w warunkach przechowalnictwa ziemniaków.

Podczas prac eksperymentalnych na bulwach ziemniaka, na podstawie zdolności zahamowania symptomów chorobowych, wyselekcjonowano konsorcjum składające się z pięciu szczepów bakterii Gram ujemnych: *Serratia plymuthica* A294, *Lellitottia amnigena* A167, *Rahnella aquatilis* H145, *Serratia rubidaea* H440 and H469. Kolejno testowałem metody konserwacji mikroorganizmów w celu zwiększenia ich przeżywalności w formie proszków do sporządzania zawiesiny wodnej WP (ang. Wettable Powders). Następnie testowałem zastosowanie formulacji do ochrony ziemniaków w 6-cio miesięcznym eksperymencie prowadzonym w warunkach przechowalniczych. Na koniec badałem wybrane cechy składników konscorcium, jak antagonizm względem bakterii powodujących mokrą zgniliznę SRP *in vitro*, oraz antagonizm poszczególnych szczepów względem pozostałych bakterii konsorcjum.

Pozyskane wyniki doprowadziły do opracowania syntetycznego konsorcjum, oraz formulacji, która może zostać wykorzystana do ochrony ziemniaków sadzeniaków w przechowalnictwie przeciw mokrej zgniliźnie.

Wyniki zostały opublikowane w trzech eksperymentalnych artykułach naukowych.

Pierwsza praca opisuje wyselekcjonowanie i ocenę aktywności syntetycznego konsorcjium mikroorganizmów. Kolejna publikacja dotyczy opracowania, stworzenia i ewaluacji formulacji konsorcjum bakteryjnego. Ostatni artykuł opisuje genomy szczepów wchodzących w skład syntetycznego konsorcjum, oraz ich cechy genetyczne wybrane pod kątem istotności dla ich aktywności przeciwdrobnoustrojowej.

Praktycznym aspektem mojej pracy doktorskiej jest przyznanie trzech patentów (dwóch Polskich, oraz jednego Europejskiego) opisujących: (i) wykorzystanie zaprojektowanego syntetycznego konsorcjum, (ii) sposobu formulacji konsorcjum w celu zwiększenia jego stabilności półkowej oraz (iii) odczynnika do liofilizacji, który został stworzony podczas tych badań.

Podsumowując, moje badania doprowadziły do opracowania i przetestowania innowacyjnego syntetycznego konsorcjium mikroorganizmów zawierającego pięć szczepów bakteryjnych, które może zostać użyte w celu ochrony ziemniaka przed bakteriami pektynolitycznymi *Pectobacterium* spp. i *Dickeya* spp w warunkach przechowalniczych. Podczas serii eksperymentów konsorcjum zostało zbadane pod względem aktywności i stabilności w formie preparatu, który może zostać wprowadzony na rynek. Dodatkowo szczepy bakteryjne konsorcjum zostały przetestowane pod kontem negatywnego wpływu na wzrost pozostałych jego składników oraz, czy ich aktywność wynika z kooperacji szczepów konsorcjum.

Przedstawione w tej pracy doktorskiej badania konsorcjów mikroorganizmów mogą pomóc w lepszym zrozumieniu interakcji pomiędzy bakteriami antagonistycznymi, zarówno w warunkach laboratoryjnych jak i w środowisku naturalnym.

1. Popular Science Introduction:

1.1. Soil microbiology

The word soil comes from Latin *solium* ("seat, chair or throne") (Rothwell 2009), and if the soil is considered a seat for anyone, it should be microorganisms. However, they do not sit around the table peacefully but rather engage in a game of thrones (Raaijmakers et al., 2009). On the one hand, soil offers its inhabitants protection from light, retains humidity, and can support sizeable microbial biomass and diversity (Nannipieri et al. 2003). On the other hand, soil can be subjected to drought and prolonged periods of low nutrient availability (Campbell, 2009). This means that the soil is a complex and ever-changing environment for microbial communities (Nannipieri et al. 2003). However, why should we concern ourselves with the ecology of these microorganisms and their associations? Especially while it seems that the diversity of microorganisms in the soil does not play a role in the decomposition of organic matter, as it was reported that both diverse and less complex communities possess similar functionalities (McGrady-Steed et al., 1997; Naeem & Li, 1997).

Soil is a house not only for microorganisms but, among other organisms, for plants which are the global base for food production (Berners-Lee et al., 2018). Therefore, it was agriculture that drove the research on this particular ecosystem in the past (Wall et al., 2019). Plants are a primary source of nutrients for microorganisms living in soil: both as decaying matter and root exudates. Root exudates are organic compounds - both primary and secondary metabolites secreted through the roots but also shoots and leaves (Vives-Peris et al., 2019). These substances can play various roles for plants, including reshaping their microbiomes (Sasse et al., 2018). Therefore, the highest microbial diversity is in the soil zone surrounding roots called the rhizosphere (Raaijmakers et al. 2009).

Because of that, the microbial community of the rhizosphere is essential for plant wellbeing as well as production. Most soil bacteria can survive in the bulk soil (soil without a plant), but they achieve the highest densities when root exudates are available in the environment (Berendsen, Pieterse, and Bakker, 2012). These microorganisms can play various ecological roles. The most crucial roles played by these soil-inhabiting microorganisms are summarised below.

1.1.1. Decomposers of organic matter

The decomposition of organic matter in soil has been the focus of scientific society for centuries, as human civilization developed mainly thanks to agriculture (Gupta 2004). An excellent example of the importance of microbial activity for human civilization is that we most often judge soil fertility based on its structure. Obviously, the first farmers did not have other methods available to verify soil quality for farming than to evaluate its humus content (Bünemann et al., 2018). Nowadays, we have more sophisticated ways to measure nutrient content, pH, electroconductivity, electrical conductivity (EC), water holding capacity, and integration of these data (He et al., 2021; Rinot et al., 2019). However, it is suggested that the main contributor to soil degradation is the deterioration of its structure (Bünemann et al., 2018). Therefore, simple organoleptic examination is still a dependable method for soil health and fertility assessment (Dhaliwal et al. 2019). It means that there is no fertile soil without microbial activity.

The most important role of microbial communities in the soil is carbon turnover, which, almost exclusively, can be performed by microorganisms **(Fig. 1)** (Adl 2004). Enormous quantities of organic carbon sequestered by plants must be recycled back to the inorganic form. Soil releases ten times more inorganic carbon into the atmosphere than anthropogenic sources altogether (Högberg and Read 2006).

Nontheless, it is no longer believed that most of the carbon absorbed by plants returns to the soil as a decaying matter but is mainly released through living roots in the form of root exudates (Högberg and Read 2006). This does not change the fact that we will soon be drowned under the pile of dead plants without bacteria and fungi that are able to decompose them. Decomposition is vital for recalculating essential elements of life and provides a particular soil structure that retains water and nutrients (Dhaliwal et al. 2019). Plant dry matter comprises primarily cellulose, lignin, hemicellulose, and secondary metabolites, all in the form of stable compounds requiring enzymatic digestion (Eldor A. Paul 2015).

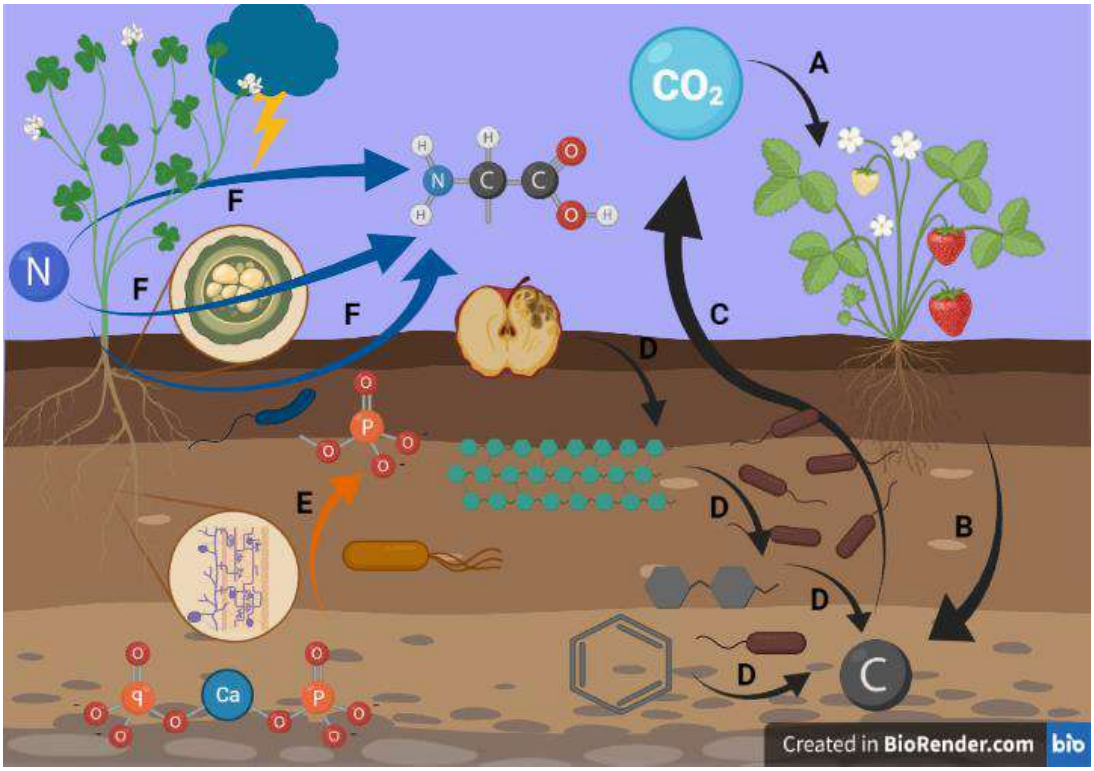

Figure 1. Microbial activity in the soil. Black arrows indicate carbon turnover, orange phosphate solubilization, and blue ones nitrogen fixation. A - carbon dioxide fixation by plants; B – organic carbon release by root exudates (most of the fixed carbon returns to atmosphere through the form of root exudates); C- release of inorganic carbon to the atmosphere due to microorganisms' metabolic activity; D – decomposition of complex organic particles by microorganisms (sometimes process requires a sequence of anerobic and aerobic processes, which can be achieved thanks to soil granular structure); E – phosphate solubilization, phosphate is the second most crucial element for agriculture production, (phosphate and other essential elements can be present in the soil but may be inaccessible by plants due to forming insoluble salts, phosphate is solubilized by bacteria through soil acidification, but fungi can solubilize phosphate without soil acidification); F – nitrogen fixation, nitrogen is the most crucial element for agriculture production (there are 3 primary natural sources of organic nitrogen for plants: 1, thunderstorms where nitrogen can be oxidized thanks to electric discharge and be trapped by falling rain, 2, oxidation by nodule forming bacteria: S*inorhizobium* spp. and *Rhizobium* spp., 3, oxidation by free living bacteria e.g. *Azospirillum* spp.)—created with BioRender.com.

Saprophytic bacteria and fungi are the masters of carbon metabolism. The wide array of secreted enzymes does not only degrade the resistant carbohydrates but also breaks down toxic compounds of both natural and anthropogenic origin (Ostrem Loss and Yu 2018). Therefore, it is unsurprising that composted plant debris is still commonly used as a fertilizer, even though it does not positively affect soil chemical properties as synthetic products. Compost, however, improves significantly soil physical properties (Celik, Ortas, and Kilic 2004).

Such practices increase soil's water holding capacity and decrease nutrient runoff (Adugna 2016). This means that even though saprotrophic microorganisms act mainly through improved soil physical properties, they can substantially improve the yield and protect the soil from the negative effect of prolonged drought and intensive precipitation. Soil microorganisms can also have more direct fertilization effects.

1.1.2. Phosphorous solubilizators

Phosphate is the second most crucial element for agricultural production after carbon. Phosphate, however, does not have an inexhaustible source as nitrogen and phosphate fertilizers are produced mainly from mined substrates. Limited deposits of phosphate and growing demand from both agriculture and the chemical industry cause increasing prices (Chien et al. 2011). Extensive usage of mineral fertilizers also leads to heavy metal accumulation in the fields, posing a severe environmental risk (Jiao et al. 2012). How do natural ecosystems deal with this limiting resource? Firstly, phosphate returns to the soil with decaying and/or dead matter in the natural ecosystem. In the case of phosphate, organic phosphate fertilizers fail to provide enough of its accessible form to the plants at their initial growth stage (Johnston et al., 2014). This situation is caused by a slow process of phosphate recruitment from organic fertilizers. This problem calls for change in currently used practices towards more sustainable ones (Childers et al. 2011).

There are several possible solutions to that problem. Firstly, we should take a deeper look into using phosphate deposits and ensure that the waste is limited. The fertilization should be planned to maximize its effectiveness and minimalize the runoff (Crombez et al., 2019). The third promising strategy is to increase soil availability for plants with microorganisms. Increased root branching facilitates nutrient uptake. The above can be achieved by selective breeding and auxin-producing bacteria (Chowdhury et al. 2017).

There are also multiple reports on the beneficial effect of mycorrhizal fungi on water and nutrient uptake (Bucher 2007; Nagy et al. 2009; Rhodes and Gerdemann 1975). Microorganisms increase the absorption rate by increasing the absorption surface and can also solubilize organic and inorganic phosphate deposits **(Fig. 1)**. The mechanism usually requires the production of organic acids. However, it has been reported that fungi can solubilize phosphate without acidification (Khan, Zaidi, and Ahmad 2014). This leads us to conclude that microorganisms can facilitate phosphate uptake by multiple mechanisms: indirectly by solubilizing inorganic phosphate salts, realizing it from soil organic matter (SOM) or reducing phosphate runoff, but also directly through symbiotic interactions with plants (Rawat et al., 2020).

1.1.3. Nitrogen sequestrators

When writing about the beneficial impact of microorganisms on plant growth, it is impossible not to mention the essential element – nitrogen. Although nitrogen molecules comprise approximately 78% of the atmosphere, nitrogen is the most limiting factor for primary production on Earth (Leghari et al. 2016). Therefore, it is forecasted that approximately 111 million tons of nitrogen fertilizers will be used in 2022 (Food and Agricultural Organization of United Nations 2017). This amount is twice the amount of phosphate fertilizers used for agricultural applications.

This vast demand could not be met by potassium nitrate deposits mining. This is caused by the fact that over 50% of the used product will not be absorbed by plants but will be lost to the atmosphere (Zhang 2017). It is the huge source of air and water pollution that contributes to global warming (Savci 2012) and plays a significant role in introducing harmful algal blooms (Heisler et al. 2008). Nevertheless, we can turn to microorganisms for ecological and financial reasons to decrease the need for artificial nitrogen fertilization.

Nitrogen fixation by nodule-forming plants is a well-known fact accepted by general publicity. The unique plant family known for its fertilization properties which come from nitrogen fixation is Legumes (*Fabaceae*). These plants are commonly used as a catch crop, grown between the main crops to reduce erosion and keep the soil fertile (Thorup-Kristensen, Magid, and Jensen 2003). However, plants cannot fix nitrogen by themselves, and they need bacteria for that. *Rhizobium* and *Sinorhizobium* spp. produce nitrogenize - an enzyme with which they can convert atmospheric nitrogen to organic one (Janssens et al. 2000). This enzyme has in its catalytic pocket the most significant iron-sulfur cluster known so far. This particle is especially prone to oxidation because of the chemical similarity between nitrogen and oxygen. Therefore, for the nitrogenize not to get deactivated, anaerobic conditions are required. It is is the role of a plant which forms nodules for nitrogen-fixing bacteria providing anaerobic conditions and organic carbon for them (Sachs, Quides, and Wendlandt 2018). Yet, nodule-forming bacteria are not the only organisms that can fix atmospheric nitrogen. Free-living bacteria have evolved various methods of protecting nitrogenase from oxidation (Janssens et al. 2000).

Azospirrillum spp. are the most studied and commonly used free-living nitrogen-fixing bacteria, though *Azospirillum* spp. do not reside in nodules; it relies mainly on creating a physical barrier for oxygen. Bacteria colonize roots and produce biofilm, reducing gas exchange with the environment (Janssens et al. 2000). These bacteria are commonly used in agriculture in South America, especially in Soybean (*Glycine max* L.) production. Many new biofertilizers containing these microorganisms have been introduced into market in recent years (Zambrano-Mendoza et al., 2021). This leads to scientific interest in this subject. We now know that they can form symbiotic interactions with plants and green algae, probably since this genus relatively recently transformed from aquatic to the terrestrial environment (Cassán et al. 2020).

A deeper understanding of the biology and ecology of microorganisms leads to new and more successful applications.

1.1.4. Mycorrhizal fungi

While discussing plant growth promotion by microorganisms, it is vital to mention mycorrhizal fungi. Fungi are found among the largest organisms on Earth since one specimen of *Armillaria ostoyae* (Romagn.) can reach 95–965 ha (Ferguson et al. 2003). Fungi are best known as saprophytes but, as in the case of *Armillaria ostoyae,* can be pathogenic and plant beneficial. Interestingly, some pathogenic fungal species have strains that may be plant beneficial (Mandeel and Baker 1991). Plant beneficial strains of fungi often infect plants similar to the pathogen. For example, arbuscules formed by Arbuscular Mycorrhizal fungi (subphylum *Glomeromycotina*) resemble haustoria formed by obligatory biotrophic pathogens (Voegele and Mendgen 2003). Furthermore, Arbuscular Mycorrhizal (AM) fungi are the most studied and used plant growth-promoting microorganisms (Adeyinka Fasusi et al. 2021).

Mycorrhizas are the most common interkingdom symbioses, but amongst them, AM is the most ancient and most widely distributed in plant taxa. It is suggested that thanks to AM, first liverworts (*Marchantiophyta*) could colonize the land (Genre et al. 2020). Less than a third of the vascular plant species cannot form AM. They belong to the families: Brassicaceae (*Brassicaceae* Burnett.), Amaranthaceae (*Chenopodiaceae* Vent.), Caryophyllacea (*Caryophyllaceae* Juss.), and Proteaceae (*Proteaceae* Juss.) (Cosme et al. 2018). This makes AM fungi a universal organism for plant growth promotion of many economically important crops. AM is known for increasing plant resistance to abiotic stress (Malhi et al., 2021). Fungal hyphae reaches from the roots penetrating the soil and growing the pool of available nutrients to the plant, therefore, it is most helpful in increasing drought tolerance (Xu et al., 2018) and low phosphate availability (Bucher, 2007).

AM fungi can, however, act as key species for biological plant protection (Xu et al., 2018). This could be an essential function, especially considering that the vascular plans evolved accompanied by AM (Genre et al., 2020). Plant and fungal symbiosis regulation suggest that they are indeed vital elements in plant microbiota communication (Parniske, 2008). AM fungi can also modulate the soil organic matter dynamics (Frey, 2019).

Why then are AM fungi not more commonly used in agriculture? Firstly, they are obligatory symbionts, which means they cannot live and reproduce without a host plant (Frey, 2019). The most promising approach to producing large quantities of selected AM fungi spores is hairy roots culture (Ho-Plágaro et al., 2018). Another vital issue is the natural occurrence of AM fungi. Since AM is so prevalent in nature, another AMF species will compete for a niche with a used inoculant. It is suggested that tillage and extensive usage of fungicides lead to a situation when fields are deprived of these beneficial microorganisms (Mang'erere Nyamwange et al. 2018). However, currently used agricultural practices seem to reduce the natural AMF inoculum. Usually, a certain level of natural infestation is present (Bernaola et al., 2018; Xavier Martins & Rodrigues, 2020).

1.1.5. Necrotrophic pathogens

Microorganisms are not always friendly toward plants, which have natural defence systems, and a healthy plant can defend itself against most pathogen attacks using innate defence immunity (Faris & Friesen, 2020). However, in agriculture, the pathogen pressure can be higher than under natural conditions, and the chance of spreading the disease is enormous. These pathogens can successfully attack the awakened plant and spread across the field. Necrotrophic pathogens such as *Botrytis cinerea* mainly attack fruits through enzymatic digestion, producing lesions (Petrasch et al., 2019).

Though most plants will likely resist the pathogen invasion and remain alive, most of the productivity will be lost. Increasing general plant health by beneficial microorganisms can decrease the chance of infection and lower adverse outcomes. This, however, is not a satisfactory result for large-scale agricultural production.

Therefore, vast amounts of fungicides (0.63kg/ha in Europe) are used in agriculture every year to tackle these pathogens. Furthermore, more fungicides are required at higher temperatures (southern Europe 1.69kg/ha) (Faris & Friesen, 2020), yet, fungicides are not specific to pathogens but can also harm humans (Faris & Friesen, 2020).

However, pathogens are not only necrotrophic. For example, probably the most known plant pathogen in history, *Phytophthora infestans*, a causing agent of a light blight, which precipitated/ led to the Great Irish Famine 1845-52, is hemibiotrophic. It means that *P. infestans* has a biotrophic as well as necrotrophic stage (Faris & Friesen, 2020). In the beginning, the pathogens infect the plant, fighting its natural defences and reducing the symptoms. In contrast, if the plant is fully infected, the pathogen can switch to the necrotrophic stage and kill the whole plant. In this case, it is not only an elaborate mechanism increasing the infection site but also allowing the pathogen to finish its lifecycle as oomycetes.

1.1.6. Biotrophic pathogens

Biotrophic pathogens form a different relationship with plants than necrotrophic ones. They are more specialized and do not often cause immediate plant death. They take their nutrients from the host's living cells, and cell necrosis is the primary plant defence mechanism applied by plant hosts against those pathogens (Glazebrook, 2005). Obligatory biotrophic pathogens depend on the plant to the extent that they cannot be cultivated without their hosts.

Fungal biotrophic pathogens form haustoria structures similar to the arbuscules formed by obligatory symbiotic *Glomeromycot*a (Panstruga, 2003). Another characteristic phenomenon for biotrophic pathogens is a gene for gene resistance (Thompson & Burdon, 1992). These pathogens need to highjack the plant metabolism for their benefit while avoiding triggering plant defence response (Glazebrook 2005). If the plant could recognize biotrophic pathogen in time, it would induce necrosis in the place of infection, preventing further spread of the disease. This is achieved by inducing the salicylic acid (SA) pathway, while resistance for necrotrophic pathogens is influenced by the jasmonic acid (JA)/ ethylene (ET) pathway (Barna et al., 2012). Therefore, biotrophic pathogens and plants are involved in a constant fight producing different virulence factors and resistance genes. This leads to the situation when some varieties are suspectable or resistant to specific pathogens (Glazebrook 2005). In turn, necrotrophic pathogens have a more continuous spectrum of resistance. However, this is only a theory since there are only a few examples of classical biotrophic or necrotrophic pathogens, and all stages in between are possible (Glazebrook 2005).

Thus, we can see that plants do not have easy lives, and the wrong decision on how to defend themselves may endanger their lives. Nevertheless, we should not worry; they have powerful allies.

1.1.7. Pathogen's antagonists

Who can be those powerful allies, and why would they protect the plant from the pathogens? Firstly, these microorganisms can count on the root exudates, a rich source of organic carbon (Högberg and Read 2006). To let the plant die is to lose a stable source of nutrients. While pathogens exploit their host to multiply quickly, these antagonists usually choose a different ecological strategy.

Ecological strategies K or r were developed as a concept for animals but can be applied for microorganisms as well, with an additional L strategy (Golovlev, 2001). Shortly, K strategy aims to develop competitiveness sacrificing the replication rate, while r strategy favours fast replication, not putting many resources into competitiveness and L strategy is aims at long-term survival in the form of spores (Kurihara et al., 1990). With this perspective, when we look at the microorganisms in the soil, we can see that some, like necrotrophic pathogens, use r strategy to multiply quickly after infecting the host. Others, in turn, can try to secure their place in the environment by creating favourable conditions producing antimicrobials and possessing genes for resistance. This strategy predisposes them to be pathogen antagonists (Golovlev, 2001).

Understanding why microorganisms would benefit from protecting plants from pathogens requires answering the question of who they are. The antagonisms play a central role in the life of bacteria in the community (Peterson et al., 2020). Thus, these microorganisms, producing a wide array of antimicrobials, can successfully colonize the plant rhizosphere and claim the exudates for themselves. However, these microorganisms must use different action modes to drive away from the competition and be prepared for the counterattack (Raaijmakers et al., 2002). Therefore, the phyla used for biological protection are known for extensive secondary metabolisms (Shoda, 2000) like *Bacillus* spp. and *Pseudomonas* spp.

1.2. Biological Plant Protection

Antagonistic bacteria, which are strong competitors, reduce the chance of the plant falling prone to the pathogen. Unsurprisingly, people would like to exploit this phenomenon in agriculture (Nega, 2014) which takes us back to the beginning. The whole concept of biological plant protection could only unfold after observing the phenomenon of suppressive soils.

The suppressive soil is the soil where the plants are less prone to infection by pathogens (Hornby, 1983). The phenomenon is similar to soil fertility but not quite the same. After a series of experiments, it was proven that suppressiveness is transferable and is attributed to the activity of microorganisms (Schroth & Hancock, 1982). Then, there was nothing left to do but find the best microorganism protecting the crops from falling, the bacterial Captain America.

The ordeal, however noble, is not an easy one. To begin with, microorganisms have different strategies and may not be effective against various plant pathogens (Bélanger et al., 2012). Also, soil type and even plant cultivars can influence the activity of an antagonist (Meyer et al., 2010). Therefore, we could look for specific antagonists called biocontrol agents (BCA's) for certain crops and diseases. However, we should also bear in mind a possible ecological threat connected with incorporating large quantities of microorganisms from distant geographical regions. Nagoya Protocol was implemented for these and, more importantly, economic reasons. It restricts the usage of microorganisms from different geographical zones (Watanabe, 2015). However, we can find BCA suitable for other conditions with enough effort and funding. The question is if these microorganisms could compete with chemical pesticides, and if so, how?

1.2.1. Competition

I would first mention competition among the different modes of action of BCA's. The rhizosphere is only a tiny space on the root surface with abundantly available nutrients, and many would like to occupy this place. Therefore, the microorganism must compete for both nutrients and space (Whipps, 1997). Although all of the following mechanisms serve to drive adversaries away, the competition *sensu stricto* is when only primary metabolism is involved.

Competition for ecological niche

It is hard to draw a clear line between competition for niche and nutrients. While bacteria occupy a particular space, they can limit access to nutrients to others and impede colonization by absorbing the necessary nutrients (Whipps, 1997). However, these two modes of action are often intertwined and are hard to separate; we can observe that they are both essential modes of action (Fravel et al., 2003). Furthermore, the root exudates are not uniform along the entire root length (Marschner, Römheld, & Kissel, 1987). Using that information, we assess if the stains will compete for niche based on their carbon source preferences (Magan & Aldred, 2008). Although the competition for niche seems to be a promising mode of action, since it seems complicated for the pathogens to overcome, it is not widely studied, and most papers regarding competition refer to nutrients. Hopefully, developing new techniques for observing rhizobacteria *in situ* will facilitate the research on that matter (Kiely et al., 2006; Singh et al., 2004).

Competition for nutrients

The other field of competition is competition for nutrients. Depending on the environment, different nutrients can be more limiting, but carbon is usually the most critical factor for microorganisms (P. Marschner, 2011). Competition for carbon can be an essential mode of action against Fusarium wilt (O'Brien, 2017) or Pythium damping-off (Elad, 1987). Yet, microorganisms do not only fight for carbon. A good example is iron, which is interesting for many reasons. Firstly, iron is usually abundant in soil but unavailable for plants and microorganisms (Colombo et al., 2014). Therefore, to absorb iron, both plants and microorganisms developed ways of solubilizing it. Microorganisms and monocots produce siderophores, compounds chelating ferric ions (Fe^{3+}).

While dicots have to reduce Fe^{3+} to Fe^{2+} state and only then could they uptake it, they could also use iron chelated by siderophores produced by monocots (Colombo et al., 2014). Microbial siderophores as potent chelators find numerous applications in industry, contributing to the scientific community's attention (Ahmed & Holmström, 2014; Saha et al., 2016). Siderophores can be used: in plant fertilizers (Barton & Abadia, 2006), as biocontrol agents (Yu et al., 2011), in the bioremediation of heavy metals (Rajkumar et al., 2010), for paper pulp bleaching (Bajpai, 2004), in optical biosensors (Yoder & Kisaalita, 2011), in medicine for drug delivery (Möllmann et al., 2009), or even in nuclear fuel reprocessing (Marshall et al., 2010). Production of siderophores is a promising mode of action because it is safe; it has a broad mode of action, but this is primary metabolism, and microorganisms produce other more potent substances suppressing microbial growth (Saha et al., 2016).

1.2.2. Production of antimicrobials

Microorganisms produce a wide array of secondary metabolites with antimicrobial activity. These compounds are fascinating to scientists since they can be used in industry (Ghosh et al., 2019). The most known example of such a chemical is penicillin, an antibiotic produced by a fungus *Penicillium* sp. was discovered by Aleksander Flaming (Fleming, 2001). This breakthrough discovery led to him being awarded the Nobel prize but, most importantly, revolutionized medicine. This potent drug could treat tuberculosis, a devastating bacterial disease that took the bloody toll. The revelation has led to the discovery of other microbial antibiotics (Gaynes, 2017). Nowadays, antibiotics are mostly synthetically produced, the compound naturally produced by microorganisms is the foundation for their design (Bérdy, 2012).

Antibiotics

First, we have to answer the question about what antibiotics are? Paul Ehrlich first used the term antibiotics to describe coloured substances with antimicrobial properties (Bérdy, 2012). The first such substance described by him was salvarsan. This substance is not of antimicrobial origin but synthetic, and it started the research in the field being the first successful drug against syphilis (Zaffiri et al., 2012). Currently, the term antibiotic means semi-synthetic or natural antimicrobial chemical substance. Although we all have a general idea of what antibiotic is, in terms of secondary metabolites, there is no strong separation between them and other substances (Maartens et al., 2011). Regardless of where we put the division between antibiotics and other chemotherapeutics, it is impossible to ignore their role in the development of medicine (Gaynes, 2017).

Soil microorganisms are a significant source of novel antibiotics (Durand et al., 2019). Although synthetic antimicrobials find wider and broader usage in medicine (Walsh, 2003), the soil is the source of the great diversity of antimicrobial production and resistance (Crits-Christoph et al., 2018). Antibiotics have an essential role for soil microbial communities, and, although they are toxic substances produced to drive the competition away, soil microorganisms learned to use them as a carbon source (Dantas et al., 2008). Even though the detected concentration of antibiotics in the soil seems to be relatively low, the ability to produce a wide array of antibiotics has proven to be a good predicament for use as BCA (Raaijmakers et al., 2002).

Bacteriocins

Another potent group of antimicrobials can be bacteriocins, ribosomal-produced peptides. The most known mode of action of bacteriocins in membrane permeabilization, but other mechanisms, such as DNA degradation, were also reported (Yang, Lin, Sung, & Fang, 2014). However, many bacteriocins lose their activity during purification and studying their mechanism of action is more complex than studying antibiotics. Furthermore, these peptides usually have a narrow spectrum of activity, limited to the closely related species (Cesa-Luna et al., 2020). Bacteriocins have found their usage in the food industry and can have an essential role in biological plant protection. Still, , as they are ribosomally-produced peptides, they are usually less stable than antibiotics, so they are not widely used in medicine (Subramanian & Smith, 2015). This obstacle can be easily overcame in agriculture if we consider using living microorganisms, since numerous bacteria known for producing bacteriocins are isolated from plants. The perfect example of this is *Bacillus* spp. The members of this genus produce a wide array of antimicrobials and multiple bacteriocins (Abriouel et al., 2011), but also the ability to produce spores by the members of this genus is beneficial for preparing formulations. Bacteriocins produced by *Rhizobacteria* can suppress the growth of pathogens and have plant growth-promoting properties, like Thuricin 17 produced by *B. thuringensis* (Nazari & Smith, 2020).

1.2.3. Hyperparasitism and predation

In the complex trophic interactions in the rhizosphere, pathogens can become prey. When we think about predation, we most probably see large animals running after smaller ones, but this interaction is not limited to megafauna and indeed finds usage in agriculture (Chailleux et al., 2014; Omkar & Pervez, 2003; Stiling & Cornelissen, 2005).

Though most of the research on the use of predation against plant pests concerns the use of insects or spiders, this phenomenon is not limited to the animal kingdom; bacteria can also be predators (Pérez et al., 2016). Predatory bacteria (mainly *Bdellovibrio* and *Bdellovibrio*-like bacteria) are, unlike other predators, smaller than their prey. They can have different "hunting" strategies from pack to single and different diverse spectrums of prey (Markelova, 2010; McNeely et al., 2017). Predation seems to be a promising mechanism for biocontrol of plant diseases and eradicating food-borne human pathogens. The lack of success in their usage in agriculture may be attributed to the problematic culture of these organisms and the feeding strategy. The generalists will also reduce the number of other beneficial bacteria. At the same time, merely specialized strains will not be able to sustain the high cell count without the presence of the preferred host (Olanya & Lakshman, 2015).

The production of lytic enzymes may also be considered as a mechanism of predation in the microbial world (Chet et al., 1990). To digest complex unsolvable compounds and use them as a source of nutrients, microorganisms have to produce and excrete lithic enzymes. Saprophytes often use these enzymes to decompose dead plant matter. They can also be used to break down the cell wall of leaving organisms like plants, bacteria or fungi (Medie et al., 2012). Therefore, the microorganisms which can degrade pathogenic fungi cell walls can be used to protect plants against them (Chet et al., 1990). Numerous bacteria species can produce chitinases, enzymes breaking down the main component of the fungal cell wall. Genera, known for producing such enzymes used for biological plant protection, are *inter alia*: *Streptomyces*, *Bacillus*, *Pseudomonas* and *Serratia* (Herrera-Estrella & Chet, 1999). Besides secreting chitinolytic enzymes, these bacteria produce a wide array of antimicrobials that allow them to successfully kill plant pathogens and use them as a source of nutrients (Veliz et al., 2017). However, the best known and widely studied

microorganisms for biological protection which use this mechanism are *Trichoderma* spp. (Viterbo et al., 2002). These microorganisms, like others, do not use only one mechanism to fight the pathogens but also produce antimicrobials, compete for nutrients, promote plant growth, induce plant resistance, penetrate the fungal cell wall, and become a parasite of many vital plants pathogens (Sood et al., 2020).

This parasitic interaction when the host is a parasite of another organism is called hyperparasithism. Hyperparastitism in the microbial world is often found in the Fungi kingdom, where one strain forms haustorium-like structures attaching itself to the host and continuously obtaining nutrients without killing it (Köhl et al., 2019). Although many fungal hyperparasites from different families have been identified so far (Hijwegen & Buchenauer, 1984; Jeffries, 1995), the majority of research concerning microorganisms with this mechanism of activity is limited to two genera, *Trichoderma* (Vinale et al., 2008) and *Clonostachys* (Nygren et al., 2018). If we do not need to kill the pathogen to stop the disease, methods may prevent them from developing the disease symptoms.

1.2.4. Disruption of pathogenesis

Pathogens that cause a disease need three things: pathogen inoculum, favourable conditions and a susceptible host. These features form the so-called "disease triangle" (Scholthof, 2007). Therefore, pathogens successfully colonize the plant, respond to the environmental conditions and express the genes responsible for their pathogenicity only in the favouring conditions (Velásquez et al., 2018). Yet, we may trick or convince the pathogens that the environment is not favourable and stop them from causing the disease.

Pathogens, to be able to produce enough enzymes to penetrate the plant cell wall and overcome the plant's defences, need to reach a specific density.

Therefore, they have developed a system of monitoring cell numbers or density in the environment, called *quorum sensing* (QS). Thanks to that mechanism, bacteria can simultaneously start producing lytic enzymes when they reach high enough inoculum to start the disease (Azimi et al., 2020). However, the quorum quenching can disrupt their communication (QQ). There are several ways of disrupting QS. Firstly QS can be silenced by degradation of QS compounds like N-acyl-homoserine lactone (AHL) (produced by Gram-negative bacteria). This is achieved chiefly by acylases (which remove the lactone ring from the acyl tail) or lactonases (hydrolysis of the lactone ring). This mechanism is often found in Gram-positive bacteria, which use peptides for QS (Prazdnova et al. 2022) primarily, and because AHLs and products of their natural degradation (tetramic acids) are toxic to them (Grandclément et al., 2015). But also Gram negative bacteria use this mechanism: *Pseudomonas* (Rodríguez et al., 2020), *Ochrobactrum quorumnocens* (Krzyżanowska et al., 2019) and many others (Jafra et al., 2006). Others can inhibit the synthesis of signal molecules, compete with its receptors or disrupt the transduction cascade (Paluch et al., 2020). All of the mentioned mechanisms may be useful for biological plant protection. As a result, to identify the most promising agents, we should identify the most important plant pathogens (Gutiérrez-Pacheco et al., 2019).

1.3. Essential plant diseases - an introduction

Plant diseases are divided by the causing agent into three major groups, viruses, fungi and bacteria (Nazarov et al., 2020). Plant viruses and viroids (infectious RNA particles without protein coat) are not living organisms and cannot actively infect the plant. Instead, they are transferred horizontally (from parent to offspring) or vertically, mainly thanks to insect or nematode vectors (Jones & Naidu, 2019). Fungi are multi or unicellular eukaryotic organisms.

They usually have complex lifestyles and can be pathogenic in a specific form while being saprotrophic in the other. Often other eucaryotic microorganisms (e.g. *Oomycota*), which are pathogenic to plants, are classified in this group, which is not taxonomically correct (Doehlemann et al., 2017). However, this approach is commonly used due to constant changes in the eucaryotic phylogenetic tree (Burki et al., 2020) and the functional similarity of these pathogens (Money, 1998).

Bacterial plant pathogens are unicellular organisms that can coordinate their activity and actively penetrate plant tissues. The virulence factors necessary for infection can be carried in their chromosome but also can be coded by mobile elements like plasmids which can be transferred horizontally or carried by bacteria infecting viruses (bacteriophages) (Sundin et al., 2016). Interestingly, there has not yet been identified any plant pathogenic Archea, which leads to a widespread assumption that these organisms cannot be pathogenic to plants or animals, even though they have the genetic potential to be pathogenic (Cavicchioli et al., 2003).

1.3.1. Important viral pathogens

Climate change and changes in agricultural practices in recent years seem to favour the spread of viral plant pathogens. Even though viruses and viroids are non-motile and depend on the environment to be spread, they can cause substantial economic losses in agriculture. Viruses can spread unassisted: by pollen, direct plant contact or by water or soil, or can be transmitted by insects, mites, protista or humans. All these ways of transportation seem to be aided by modern agriculture practices (Jones & Naidu, 2019). The growing number of plant viral epidemics can be partially attributed to climate change, but the major contributor seems to be international trade. On the heavily populated Earth, when food consumption continuously increases, the international vegetable trade is essential to maintaining food security (Jones, 2021).

To minimize the spread of the diseases by food transport, plants and seed material must be checked before being exported. However, due to globalization, humans have become the vectors of important animal and plant viruses (Ranawaka et al., 2020). The most important plant viruses are in descending order: Tobacco mosaic virus (TMV), Tomato spotted wilt virus (TSWV), Tomato yellow leaf curl virus (TYLCV), Cucumber mosaic virus (CMV), Potato virus Y (PVY), Cauliflower mosaic virus (CaMV), African cassava mosaic virus (ACMV), Plum pox virus (PPV), Brome mosaic virus (BMV), Potato virus X (PVX) (Scholthof et al., 2011). Since plant viruses are primarily transmitted between plants via insects or mites, managing these diseases relies on insecticides (Castle et al., 2009).

1.3.2. Important fungal pathogens

Although fungi were the first to be recognized as critical plant pathogens, they have not lost their significance, and their influence is still underestimated (Fausto et al., 2019). Fungi and oomycetes not only pose a threat to cultivated but also stored plant material and produce potent mycotoxins, which can cause severe poisoning in humans and animals (Fisher et al., 2020). Fungi are incredibly efficient plant colonizers, whether they are plant pathogenic or beneficial strains. Their tight interactions with plants are extensively studied for their beneficial and harmful influence on the crops (Chen et al., 2019). It is suggested that fungi present in the environment can quickly adapt to the changing environment and emerge as pathogens of plants in monocultural agricultural systems (Giraud et al., 2010). The most important fungal plant pathogens are, in descending order: *Magnaporthe oryzae*, *Botrytis cinerea*, *Puccinia* spp., *Fusarium graminearum*, *F. oxysporum*, *Blumeria graminis*, *Mycosphaerella graminicola*, *Colletotrichum* spp., *Ustilago maydis*, *Melampsora lini* (Dean et al., 2012).

The most important plant pathogenic oomycetes are, in descending order: *Phytophthora infestans*, *Hyaloperonospora arabidopsidis*, *P. ramorum*, *P. sojae*, *P. capsica*, *Plasmopara viticola*, *Phytophthora cinnamomic*, *P. parasitica*, *Pythium ultimum*, *Albugo candida* (Kamoun et al., 2015). The losses caused by these pathogens have led to extensive usage of fungicides in the field and in storage, which can have a deleterious effect on the environment, and human health (Faris & Friesen, 2020), but also cause increasing resistance (Lucas et al., 2015). Therefore, it is essential to develop alternative approaches to control the spread of fungal diseases (Baibakova et al., 2019).

1.3.3. Important bacterial pathogens

Bacteria like fungi cause substantial economic losses in the field and the storage of essential crops (Nazarov et al., 2020). Additionally, the recent outbreaks of human bacteria poisoning from fresh plant produce have caught scientific attention (Berger et al., 2010). It seems that some human pathogens cannot only survive on plant material but also colonize plants and plant produce (Lim et al., 2014). Human pathogens can also be pathogenic to plants (Kirzinger et al., 2011). However, they are not the most economically significant plant pathogens; this cross-kingdom pathogenicity illustrates the huge adaptive potential of bacteria. The most critical bacterial plant pathogens are, in descending order: *Pseudomonas syringae, Ralstonia solanacearum*, *Agrobacterium tumefaciens*, *Xanthomonas oryzae* pv. oryzae, *X. campestris*, *X. axonopodis*, *Erwinia amylovora*, *Xylella fastidiosa*, *Dickeya dadantii* and *D. solani*, *Pectobacterium carotovorum* and *P. atrosepticum* (Mansfield et al., 2012). Thanks to their genomic plasticity, bacteria can quickly adapt to changing conditions by acquiring virulence factors necessary for plant colonization (Kado, 2009) or gaining antibiotic resistance (Sundin & Wang, 2018). Bacteria are a broad and diverse group of microorganisms and since they differ in their biology, so differ the approaches to fighting them.

For example, *Candidatus* Liberibacter asiaticus requires a psyllid vector for the spread and all the attempts to control these pathogens are concentrated on its vector (Andrade et al., 2020), while devastating, *Clavibacter michiganensis* is a seed-born pathogen (Nandi et al., 2018).

Potato Soft Rot is a disease of particular interest, with the growing incidence in temperate climate regions. It is caused by two bacteria groups included in the top 10 most critical bacterial pathogens: *Pectobacterium* spp. and *Dickeya* spp. (Mansfield et al., 2012).

1.3.4. Potato Soft Rot

Potato Soft Rot is caused by a group of bacteria called Soft Rot *Pectobacteriaceae* (SRP) (*Pectobacterium* spp. and *Dickeya* spp.). Its symptoms are the rotting of vegetables or ornamental plants during storage (Charkowski, 2018). The pathogens are spread mainly by progeny tubers (Pérombelon, 1974) since still many countries do not monitor this disease in seed material production (Charkowski, 2018). SRP can also be spread by air (Pérombelon, 1992), water (Cappaert, 1988) soil (Czajkowski et al., 2010), insects (Rossmann et al., 2018) and weeds (Fikowicz-Krosko et al., 2017). Since 2004, an outbreak of *Dickeya solani* has been observed in central Europe (Hadizadeh et al., 2019), and since then, it has been repeatedly spotted in the temperate regions (Potrykus et al., 2016). *Dickeya solani* is more virulent than other SRP, producing high quantities of cell wall degrading enzymes and can cause severe losses during potato storage (Czajkowski et al. 2013). Since the methods of fighting Potato Soft Rot rely mainly on sanitation practices and prevention, biological plant protection seems to be a promising approach to protecting potatoes in storage (Czajkowski et al., 2011).

2. Publications:

Here, I have enclosed three scientific articles published during my doctoral project. They describe the discovery of the artificial consortium of Gram-negative bacteria with potential activity against Soft Rot Disease, formulation and further test on the formulated bacteria in storage conditions and finally manuscript describing genetical features of strains comprising the consortium. This series of articles describes the process of creating a new biocontrol product from the idea to the biocontrol product with the potential to be used in agriculture.

2.1. Compatible mixture of bacterial antagonists developed to protect potato tubers from soft rot caused by *Pectobacterium* spp. and *Dickeya* spp [1]

[1] Reprinted from: Dorota M. Krzyzanowska, Tomasz Maciag, Joanna Siwinska, Marta Krychowiak, Sylwia Jafra, and Robert Czajkowski, 2019, Compatible mixture of bacterial antagonists developed to protect potato tubers from soft rot caused by Pectobacterium spp. and Dickeya spp, Plant disease, 103(6):1374–1382.

Short description

This manuscript describes the selection process of microorganisms and the development of the composition of strains based on their activity against soft rot disease. Here we present the development of an artificial microbial consortium instead of the most widely used single strains, which is suggested to have more repeatable activity (Sellitto et al., 2021). Additionally, to start testing biocontrol agents on the target system, not discard the strains with high activity *in vivo* but low activity *in vitro* (Köhl et al., 2011).

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e-Xtra*

Compatible Mixture of Bacterial Antagonists Developed to Protect Potato Tubers from Soft Rot Caused by *Pectobacterium* spp. and *Dickeya* spp.

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Abstract

Possibilities to protect potato tubers from rotting caused by Soft Rot Pectobacteriaceae (SRP) under disease favoring conditions were investigated using compatible mixtures of bacterial antagonists and tested with a newly developed stepwise efficacy-based screening protocol. Twenty-two bacterial antagonists were evaluated against a combination of five *Pectobacterium* and *Dickeya* strains representing species and subspecies most often associated with potato soft rot in Europe. To enable potential synergistic activity, the antagonists were initially tested against the combination of pathogens in 15 random mixtures containing up to 5 antagonists each. Three mixtures (M2, M4, and M14) out of 15 tested reduced tuber tissue maceration due to soft rot. The individual antagonists derived from M2, M4, and M14 mixtures were tested on potato slices and whole tuber injection assays. These five strains (*S. plymuthica* strain A294, *E. amnigenus* strain A167, *R. aquatilis* strain H145, *S. rubidaea* strain H440, and *S. rubidaea* strain H469) were combined to develop a tailored biological control mixture against potato soft rot. The new mixture, designated the Great Five (GF), was tested on seed potato tubers vacuum infiltrated with antagonists and subsequently with the combination of five SRP pathogens. In these experiments, the GF mixture provided stable protection of inoculated potato tubers, reducing soft rot by 46% ($P = 0.0016$) under high disease pressure conditions. The A294, A167, H145, H440, and H469 antagonists were characterized for features important for viable commercial applications including growth at different temperatures, resistance to antibiotics, and potential toxicity toward *Caenorhabditis elegans*. The implications for control of soft rot caused by SRP with the use of the GF mixture of antagonists are discussed.

Soft rot caused by pectinolytic Soft Rot Pectobacteriaceae (SRP) (formerly pectinolytic *Erwinia* spp.) (Adeolu et al. 2016), namely *Pectobacterium* spp. and *Dickeya* spp., is an important potato disease resulting in economic losses in (seed) tuber production worldwide (Pérombelon 2002). Under disease favorable conditions, *Pectobacterium* spp. and *Dickeya* spp. can cause a variety of symptoms on potato including pre-emergence decay of seed tubers, stem rot (blackleg) of field-grown plants, and soft rot of progeny tubers in storage (Pérombelon 2002). In Europe, soft rot disease results in high economic losses, mainly due to declassification and rejection of seed lots (Toth et al. 2011).

It is well established that latently infected potato (seed) tubers are the primary source of SRP (Pérombelon 1974; Pérombelon and Lowe 1975). The latent inoculum within tubers is transferred both between fields and between growing seasons (Charkowski 2006). Consequently, production of pathogen-free seed lots is considered as the most important strategy in controlling the spread of *Pectobacterium* spp. and *Dickeya* spp. in the potato ecosystem (Czajkowski et al. 2011). Pathogen-free seed tubers have been successfully produced from axenic planting material (Gopal et al. 1998). However, the use of initially clean, pathogen-free seed is of little protection as tuber contamination usually occurs in the following stages of potato seed multiplication (Charkowski, 2015; van der Wolf et al. 2017). Contamination can occur in the field during plant growth, at harvest and seed grading, as well as in storage and transit. The pathogens can also be transmitted *via* air, water, and by animals (mostly insects) entering the potato fields (Toth et al. 2003, 2011). The contamination may be internal, in the plant vascular system, in tuber lenticels and on the periderm, as well as in wounds incurred during handling. These niches are known to support long-term survival of the soft rot bacteria (van der Wolf and De Boer 2007). It is believed that wounds and cracks on the surface of potato tubers in particular, are easily invaded by the pathogens during handling and postharvest, and hence play an important role in the dissemination of SRP from a few rotting tubers to numerous neighboring healthy ones (Pérombelon 2002; van Vuurde and De Vries 1994).

Management of soft rot in potato tubers is difficult due to the widespread contamination, lack of resistance in commercial potato cultivars, and the absence of effective disease control agents (Czajkowski et al. 2011). Current integrative management strategies include the use of certified *Pectobacterium* spp. and *Dickeya* spp.-free seed tubers, hygienic measures to avoid introduction and dissemination of

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The mixture of bacterial isolates described herein, for protection of potato tubers and ornamental plants against soft rot caused by pectinolytic *Pectobacterium* spp. and *Dickeya* spp., is the object of the patent application P.423806, which has been filed with the Polish Patent Office by University of Gdansk, Poland with inventors Robert Czajkowski, Dorota M. Krzyzanowska, Tomasz Maciag, Joanna Siwinska, and Sylwia Jafra.

*The *e*-Xtra logo stands for "electronic extra" and indicates that three supplementary figures and three supplementary tables are published online.

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the bacteria, and avoidance of tuber wounding and oxygen depletion as a result of tubers becoming wet, which could further impair tuber resistance. Unfortunately, the use of integrative management has not yet led to an acceptable reduction of soft rot incidences in (seed) tubers (Czajkowski et al. 2011; Pérombelon 1992).

Biological control based on the use of antagonistic bacteria is potentially a promising alternative to or a complementation of the integrative management strategy currently employed in potato production. In addition to their antibiotic potential, antagonistic bacteria occupying the same niche may prevent colonization of (seed) tubers by *Pectobacterium* spp. and *Dickeya* spp. and consequently the development of soft rot symptoms. Some of these antagonistic bacteria are endophytes able to colonize plants systemically (Lodewyckx et al. 2002). Their potential to colonize the inoculated plant internally could provide control against pectinolytic bacteria in locations inaccessible to traditional chemical and physical control measures (e.g., vascular tissue) (Czajkowski et al. 2012b).

One of the major factors limiting the efficiency of microbial-based biocontrol agents in potato is that usually a single biocontrol agent (microbial strain) is used for both tuber and foliage diseases, moreover selected against a narrow spectrum of pathogenic strains (Diallo et al. 2011). Furthermore, most attempts do not venture beyond in vitro laboratory assays for antagonism, small-scale pathogenicity assays on tuber fragments, or tests involving culture tube-raised potato plants (Kaur and Mukerji 1999; Mota et al. 2017; Pal and McSpadden Gardener 2006). Only a few studies have included trials on a large scale near commercial situations of plant growth and storage conditions (Czajkowski et al. 2012a, b). Similarly, no large-scale experiments have been performed so far to assess the effect of mixtures containing a combination of different antagonistic bacteria to be used against *Pectobacterium* spp. and *Dickeya* spp. in potato. It has been reported in the case of other crops and their pathogens that compositions containing several biological control agents with different modes of action have superior performance over individual agents in suppressing disease symptoms (Raupach and Kloepper 1998; Stockwell et al. 2011). It can be hypothesized, therefore, that in the case of control of potato soft rot, a mixture of antagonistic bacteria would provide better protection than can be obtained with an individual biocontrol agent.

The purpose of this study was to develop a mixture of bacterial antagonists to be applied to pathogen-free (seed) potato tubers to protect them against soft rot caused by a mixture of *Pectobacterium* spp. and *Dickeya* spp. In Europe, at least five SRP species have been identified to cause potato soft rot, often present as mixed inocula (van der Wolf et al. 2017). It is well accepted that single antagonists often are unable to protect plants against multiple SRP strains due to their narrow range of antagonistic activity (Baker 1987; Czajkowski et al. 2011; Köhl et al. 2011). It was therefore crucial to develop a mixture of antagonists expressing synergistic effect and active against multiple SRP pathogens. Likewise, we aimed also to characterize the resulting mixture and its components for features important for the development of a biological control agent for large-scale commercial applications.

Materials and Methods

Bacterial strains, plant material and media. Bacterial strains used in this study are listed in Table 1. All strains were routinely grown on Tryptone Soya Agar (TSA, Oxoid) or in Tryptone Soya Broth (TSB, Oxoid) at 28°C, the latter with shaking (200 rpm), for 24–48 h. For long-term storage, bacterial strains were kept in 40% glycerol (vol/vol) at –80°C. Certified, SRP-free potato tubers cv. Irga susceptible to *Pectobacterium* spp. and *Dickeya* spp. (Salaman 2014) were used in all experiments in which bacterial strains were tested on plant material. They were purchased from Pomorsko-Mazurska Hodowla Ziemniaka (Pomeranian-Masurian Potato Breeding) (http://www.pmhz.pl/) (Szyldak, Poland).

Development of mixtures containing bacterial strains with antagonistic potential against SRP pathogens. Twenty-two bacterial strains previously studied in our laboratory showing antagonistic activity against *Pectobacterium* spp. and/or *Dickeya* spp. (Table 1) were randomly distributed to 15 mixtures each containing up to 5 antagonistic strains (Supplementary Table S1). The mixtures included strains expressing different modes of action against at least one strain of the target pathogens (*Pectobacterium* spp. and *Dickeya* spp.) (results not shown). The resulting mixtures (named: M1–M15) were subsequently tested against a combination (named: P) of five SRP strains (three pectinolytic *Pectobacterium* spp.: *P. atrosepticum* strain SCRI 1043 (Bell et al. 2004), *P. carotovorum* subsp. *carotovorum* strain Ecc71 (Willis et al. 1987), and *P. parmentieri* strain SCC3193 (Pirhonen et al. 1991) and two *Dickeya* spp.: *D. solani* strain IPO2222 (van der Wolf et al. 2014) and *D. dianthicola* strain CFBP 1200 (IPO1741) (Samson et al. 2005)). These SRP strains belong to species and subspecies most-commonly causing soft rot and blackleg diseases on potato in Europe. Although infections by multiple SRP strains may be rare in nature, these experiments were designed to simulate the worst-case scenario, in which potato tubers were infected with all five SRP strains (*P. atrosepticum* strain SCRI 1043, *P. carotovorum* subsp. *carotovorum* strain Ecc71, *P. parmentieri* strain SCC3193, *D. solani* strain IPO2222, *D. dianthicola* strain IPO1741) at high inoculum and incubated under disease-favorable conditions (high humidity, high temperature) as advised earlier (Charkowski 2015; Czajkowski et al. 2012a). Each mixture of antagonists contained an equal ratio of individual strains, with 10^8 CFU (colony forming units) ml^{-1} per strain. This amounted a total of $n \times 10^8$ CFU ml^{-1}, where n is the number of antagonistic strains in a given mixture. The suspension of five *Pectobacterium* spp. and *Dickeya* spp. pathogens contained in total 10^6 CFU ml^{-1} (2×10^5 CFU ml^{-1} of each pathogenic strain). All bacterial mixtures were prepared in running tap water directly before use.

Evaluation of mixtures containing bacterial antagonists against SRP on vacuum-infiltrated potato tubers. Inoculation of potato tubers with antagonistic and pathogenic bacterial strains was done using a vacuum infiltration method as previously described (Czajkowski et al. 2012a). Briefly, antagonistic bacterial strains were grown separately on TSA plates at 28°C for 24 h. Cells were harvested from agar plates and suspended in tap water to obtain cell density of ca. 10^8 CFU ml^{-1} (ca. 3 McF) of each strain. For this purpose, suspensions of individual antagonists with turbidity of 15 McF (measured for 10× diluted suspensions: 1.5 McF) were mixed in an equal ratio. Pathogenic strains *viz. P. atrosepticum* strain SCRI 1043, *P. carotovorum* subsp. *carotovorum* strain Ecc71, *P. parmentieri* strain SCC3193, *D. solani* strain IPO2222, and *D. dianthicola* strain CFBP 1200 (IPO1741) were grown separately on TSA and collected under the same conditions as described above, but the final density of the suspension, containing equal ratio of each strain, was adjusted to a total of 10^6 CFU ml^{-1} (0.03 McF). Certified seed potato tubers cv. Irga (Pomorsko-Mazurska Hodowla Ziemniaka, Poland) were washed under running tap water, surface-sterilized for 20 min in 5% commercial bleach solution in water, washed 3 times in running tap water, and dried in air. The tubers were then immersed in the antagonist suspension and vacuum infiltrated for 10 min at –80 Bar in a desiccator followed by 10 min incubation in the same suspension at atmospheric pressure to allow the bacteria to penetrate the lenticels and wounds of tubers. For the control treatments, potato tubers were vacuum infiltrated with tap water. Tubers were dried overnight and the next day they were vacuum infiltrated, under the same conditions as described above, with a combination of pectinolytic bacteria or with tap water (negative control). Inoculated potato tubers were placed in humid boxes (85 to 90% relative humidity), 10 tubers of the same treatment per box. Samples were incubated at 28°C for 5 days for soft rot symptom development. In each experimental run, 30 tubers (3 boxes, each containing 10 tubers) were used to test a single combination. The final experiments with a tailored mixture of antagonists were performed in 4 biological replicates (n = 120 tubers). The symptoms on each tuber were assessed using a six-rank disease severity scale developed in this study: *rank 0* – no symptoms observed on the analyzed tuber, *rank 1* – rotting symptoms localized only superficially (at the periderm) and overall on less than 25% of tuber surface, *rank 2* – symptoms observed in the rank 1 but present on 25 to 50% of tuber surface, *rank 3* – symptoms observed in the

rank 2 but additionally the tuber periderm is detached from the tuber internal tissues and rotting occupies between 50 and 90% of the tuber, *rank 4* – symptoms observed in the rank 3 but overall the rotting occupies more than 90% of the tuber surface and/or reaching the tuber internal tissue (core), *rank 5* – whole tuber macerated (Fig. 1A). The correlation of disease severity ranks in the established scale with the average potato tuber weight loss due to soft rot was evaluated in a separate experiment and prior to other tests (Fig. 1B).

Evaluation of biocontrol potential of individual antagonistic strains derived from selected random mixtures. The mixtures of antagonists that showed the best protection effect against soft rot in the initial experiment served as the sources of individual antagonistic strains for the follow-up assays. The selected strains were individually retested for their protective activity against a combination of five *Pectobacterium* spp. and *Dickeya* spp. For this, a potato slice assay was used as well as whole tuber injection assay, performed as previously described (Czajkowski et al. 2010, 2017; Maher and Kelman 1983).

Density dependence of the control of pectinolytic bacteria by a mixture of antagonistic strains on vacuum-infiltrated potato tubers. The influence of cell density on the biocontrol potential of the mixture of bacterial antagonists was evaluated on vacuum-infiltrated tubers using a similar experimental setup as described above. Potato tubers (n = 30 per treatment) were vacuum infiltrated with mixtures containing different densities of the antagonists (10^7, 10^8, and 5×10^8 CFU ml^{-1}) and a mixture of five *Pectobacterium* spp. and *Dickeya* spp. (10^6 CFU ml^{-1}) and incubated under the same conditions as described above. The experiment was independently repeated once with the same setup. Disease symptoms were evaluated using the six-rank disease severity scale.

Sequencing of 16S rDNA gene to assign antagonistic strains to bacterial species and allocation of species to risk groups. Isolation of genomic DNA and sequencing of the 16S rDNA gene (fragments ≥1,400 bp) were outsourced to BaseClear B. V. (The Netherlands). The strains were classified to the species level based on a BLAST search (Altschul et al. 1990) against the GenBank database (https://www.ncbi.nlm.nih.gov/) as previously described (Czajkowski et al. 2012a). The ATTC (American Type Culture Collection, www.attc.org) database was used to classify antagonistic strains into risk categories on the basis of their ability to cause infections in humans, animals, and plants.

Growth of antagonistic bacterial strains at different temperatures. The growth of each antagonistic strain was tested in liquid medium (TSB) over a range of four temperatures: 28, 37, 40, and 42°C and on solid medium (TSA) over a range of six temperatures: 7, 10, 28, 37, 40, and 42°C. The growth in liquid medium was assessed every hour for the total time of 16 h as previously described (Czajkowski et al. 2017). To monitor the growth of antagonistic strains on solid

Table 1. List of antagonistic isolates used in this study with reported antagonistic potential against members of Soft Rot *Pectobacteriaceae* (SRP). Antagonistic isolates developed into the final GF mixture (A294, H145, A167, H440, and H469) are marked in bold.

No.	Genus/ species	Strain	Characteristic	Biocontrol effect against	Source	Reference
1	*Bacillus cereus sensu lato*	P368	AHL inactivation, biosurfactant and siderophore production	*Pectobacterium* spp. and *Dickeya* spp. on potato	Tomato rhizosphere (Poland)	(Krzyzanowska et al. 2012b)
2	*Bacillus cereus sensu lato*	P369	AHL inactivation	*Pectobacterium* and *Dickeya* spp. on potato tubers	Tomato rhizosphere (Poland)	(Krzyzanowska et al. 2012b)
3	*Bacillus subtilis*	MB73/2	Antibiosis against SRP, biosurfactant production	*Dickeya* sp. on potato tubers	Rhizosphere of meadow weeds (Zulawy region, Poland)	(Krzyzanowska et al. 2012b)
4	*Bacillus subtilis*	P48	Antibiosis against SRP, biosurfactant and siderophore production	*Pectobacterium* and *Dickeya* spp. on potato tubers	Tomato rhizosphere (Poland)	(Krzyzanowska et al. 2012a)
5	*Bacillus subtilis*	MB5	Antibiosis against SRP, biosurfactant production	*Dickeya* sp. on potato tubers	Rhizosphere of meadow weeds (Zulawy region, Poland)	(M. Obuchowski, unpublished)
6	*Bacillus subtilis*	MB5/1	Antibiosis against SRP	n.d.[a]	Rhizosphere of meadow weeds (Zulawy region, Poland)	(M. Obuchowski, unpublished)
7	*Bacillus subtilis*	MB8212	Antibiosis against SRP, biosurfactant production	n.d.	Rhizosphere of meadow weeds (Zulawy area, Poland)	(M. Obuchowski, unpublished)
8	*Bacillus subtilis*	MB41	Antibiosis against SRP, biosurfactant production	n.d.	Rhizosphere of meadow weeds (Zulawy area, Poland)	(M. Obuchowski, unpublished)
9	*Bacillus thuringiensis*	A98	AHL inactivation, biosurfactant production	*P. parmentieri* strain SCC3193 and P. carotovorum subsp. carotovorum Ecc71 on potato tubers	Potato rhizosphere (The Netherlands)	(S. Jafra, unpublished)
10	*Bacillus weihenstephanensis*	P10	AHL inactivation, biosurfactant production	*Pectobacterium* and *Dickeya* spp. on potato	Tomato rhizosphere (Poland)	(Krzyzanowska et al. 2012b)
11	*Delftia acidovorans*	A207	AHL inactivation	*P. parmentieri* strain SCC3193 and *P. carotovorum* subsp. *carotovorum* Ecc71 on potato tubers	Potato rhizosphere (The Netherlands)	(Jafra et al. 2006)
12	***Enterobacter amnigenus***	**A167**	**AHL inactivation**	***P. parmentieri* strain SCC3193 and *P. carotovorum* subsp. *carotovorum* Ecc71 on potato tubers**	**Potato rhizosphere (The Netherlands)**	**(Jafra et al. 2006)**

(*Continued on next page*)

[a] n.d. = not determined.

medium, 2-μl aliquots of 50 times diluted in TSB overnight bacterial cultures grown in TSB were placed on the surface of TSA plates and incubated for a total period of 120 h. The growth of each strain was investigated every 24 h and rated using the following index: '–' – no visible growth, '+/–' – slow visible growth (small colonies), '+' – visible regular growth. The growth of each strain was analyzed in two replicates and the entire experiment was repeated once with the same setup.

Antibiotic susceptibility of selected antagonistic strains. The antibiotic susceptibility of selected antagonistic strains was determined by a disc diffusion method as previously described (Bauer et al. 1966). The antibiotic discs (all from BD BBL - Sensi-Disc antimicrobial test discs) used in this study were: fusidic acid (10 μg), oxacillin (1 μg), rifampicin (5 μg), aztreonam (30 μg), chloramphenicol (30 μg), imipenem (10 μg), ciprofloxacin (5 μg), linezolid (10 μg), doxycycline (30 μg), tigecyline (15 μg), streptomycin (300 μg), Synercid (4.5 μg quinupristin +10.5 μg dalfopristin), gentamicin (10 μg), ampicillin (10 μg), clindamycin (2 μg), fosfomycin (200 μg), colistin (10 μg), ceftazimide (10 μg), piperacillin+tazobactin (30 μg + 6 μg), ampiclin+sulbactan (10 μg + 10 μg), and vancomycin (5 μg).

***Caenorhabditis elegans* survival assay.** Liquid killing assay (Kirienko et al. 2014) was employed to assess the putative pathogenicity of the selected antagonistic strains to *C. elegans*. The wild-type Bristol N2 strain of *C. elegans* obtained from the *Caenorhabditis* Genetic Center (CGC) was cultured on Nematode Growth Medium (NGM) plates with the lawn of *Escherichia coli* strain OP50 and at 25°C according to the protocol described previously (Stiernagle 2006). For the killing assay, nematodes and their eggs were harvested from the NGM plates by washing the media surface with distilled water and collecting the liquid. The resulting suspension was treated with 5% bleaching solution to isolate eggs and to synchronize culture for further growth. After 48 h, when all nematodes achieved L4 larval stage, fluorodeoxyuridine (final concentration 50 μM) (Sigma) was added to liquid killing medium (LKM) to prevent the reproduction of nematodes. Cocultures of *C. elegans* and bacterial strains were performed in 48-well plates (Falcon). 100 μl aliquots of *C. elegans* culture containing approx. 30 nematodes each were placed per well and then supplemented with 100 μl of bacterial inoculum (0.5 McF, ca. 10^7 CFU ml^{-1}) in LKM. Plates were incubated for 3 days at 25°C, and the number of living nematodes was determined as described earlier (Stiernagle 2006) daily using MZ10F stereomicroscope (Leica). *Pseudomonas aeruginosa* strain PA14, with known killing potential to *C. elegans* (Tan et al. 1999), was used as a positive control. For negative control, the nematodes were grown in a medium supplemented with *E. coli* OP50. The experiment was done on three biological replicates, each containing three technical repetitions.

Statistical analyses. All statistical analyses concerning potato tuber protection assays were conducted with R version 3.3.2 (R Core

Table 1. (*Continued from previous page*)

No.	Genus/ species	Strain	Characteristic	Biocontrol effect against	Source	Reference
13	*Erwinia persicinus / Serratia rubidaea*	H452	AHL inactivation, siderophore production	*D. solani* strain IPO2019 on hyacinth bulbs	Hyacinth bulb cv. Aiolas, scale leaves (The Netherlands)	(Jafra et al. 2009)
14	*Ochrobactrum* sp.	A44	AHL inactivation	*P. parmentieri* strain SCC3193 and *P. carotovorum* subsp. *carotovorum* Ecc71 on potato tubers	Potato rhizosphere (The Netherlands)	(Jafra et al. 2006)
15	*Pseudomonas donghuensis*	P482	Antibiosis against SRP, biosurfactant and siderophore production	*Pectobacterium* sp. on potato and *D. solani* on chicory	Tomato rhizosphere (Poland)	(Krzyzanowska et al. 2012b)
16	*Pseudomonas* sp.	P486	Antibiosis against SRP, biosurfactant and siderophore production	*Pectobacterium* spp. and *Dickeya* spp. on potato	Tomato rhizosphere (Poland)	(Krzyzanowska et al. 2012b)
17	*Pseudomonas* sp.	P103	Antibiosis against SRP, biosurfactant and siderophore production	*Pectobacterium* spp. and *Dickeya* spp. on potato	Tomato rhizosphere (Poland)	(Krzyzanowska et al. 2012b)
18	***Rahnella aquatilis***	**H145**	**Antibiosis against SRP, siderophore production**	***D. solani* strain IPO2019 on hyacinth bulbs**	**Hyacinth bulb cv. Delft Blauw, basal plate (The Netherlands)**	**(Jafra et al. 2009)**
19	*Rhodococcus erythropolis*	A185	AHL inactivation	*P. parmentieri* strain SCC3193 and P. carotovorum subsp. carotovorum Ecc71 on potato tubers	Potato rhizosphere (The Netherlands)	(Jafra et al. 2006)
20	***Serratia plymuthica***	**A294**	**Antibiosis against SRP, biosurfactant and siderophore production**	***P. parmentieri* strain SCC3193 and *P. carotovorum* subsp. *carotovorum* Ecc71 on potato tubers**	**Potato rhizosphere (The Netherlands)**	**(Jafra et al. 2006)**
21	***Serratia rubidaea***	**H440**	**AHL inactivation, biosurfactant and siderophore production**	***D. solani* strain IPO2019 on hyacinth bulbs**	**Hyacinth bulb cv. Aiolas, scale leaves (The Netherlands)**	**(Jafra et al. 2009)**
22	***Serratia rubidaea***	**H469**	**Antibiosis against SRP, siderophore production**	***D. solani* strain IPO2019 on hyacinth bulbs**	**Hyacinth bulb cv. Aiolas, scale leaves (The Netherlands)**	**(Jafra et al. 2009)**

Team 2016; https://www.R-project.org/) using the RStudio (RStudio Team 2016; https://www.rstudio.com/) and the PCMR package (Pohlert 2014). The normality of data were assessed by Shapiro-Wilk test (Shapiro and Wilk 1965). Nonparametric Kruskal–Wallis test (Kruskal and Wallis 1952) was used to determine the differences between samples. For analyzing the differences between specific sample pairs, the Dunn's post hoc test was applied (Dunn 1964). Sample sizes and *P* values for each analysis are indicated in the respective figure captions.

For the *C. elegans* liquid killing assay, the survival rate, calculated over the whole experimental time, was statistically analyzed using ANOVA (analysis of variance). Results were considered to be significant at $P < 0.05$, and pair-wise differences were obtained using the *t* test.

Results

Development of the six-rank disease severity scale to assess soft rotting of potato tubers artificially inoculated with SRP. Two hundred and ten certified pathogen-free seed tubers cv. Irga were inoculated with a mixture of five pectinolytic bacteria by vacuum infiltration and kept in humid boxes, 10 tubers per box ($n = 21$), under disease favorable conditions. At the end of the experiment, each tuber was assigned a particular rank depending on the extent of soft rot symptoms (Fig. 1A). These data were compared with the average tuber weight loss (by removing rotted tissue) per box (21 boxes, 10 tubers each). The correlation coefficient (R^2) of disease severity ranks in the established six-rank disease severity scale with the average potato tuber weight loss due to soft rot was 0.9343 (Fig. 1B).

Preliminary evaluation of mixtures containing antagonistic bacteria for the protection effect on potato tubers against SRP. In the preliminary evaluation, 15 mixtures each containing up to 5 randomly selected antagonistic strains were tested for their protective effect on potato tubers vacuum infiltrated with a mixture of three soft rot *Pectobacterium* spp. (*P. atrosepticum*, *P. carotovorum* subsp. *carotovorum*, *P. parmentieri*) and two *Dickeya* spp. (*D. solani* and *D. dianthicola*) (five pathogens in total) under disease favorable conditions (28°C and 85–90% relative humidity). Three of the tested mixtures, designated M2 (containing strains: MB73/2, A207, A44, H145, and H469), M4 (containing strains: P368, P486, A167, and H440), and M14 (containing strains: A207, P103, A294, H145, and H469), reduced tuber maceration in comparison with the positive control (potato tubers inoculated with soft rot pathogens alone). Antagonistic bacterial strains present in the M2, M4, and M14 mixtures have been selected for the follow-up experiments. No protective effect was observed in the case of the 12 other tested mixtures (data not shown).

Selection of individual antagonistic strains from preliminary random mixtures and their evaluation in potato slice assay and tuber injection assay. The assay was performed to determine which individual antagonistic strains, occurring randomly in the mixtures M2, M4, M14 and selected as described above, are the most active against a mixture of SRP pathogens under disease favorable conditions. The selected mixtures contained, in total, 11 different strains: P368, MB73/2, A207, A44, P103, P486, A294, A167, H145, H440, and H469. Both the mixtures and their 11 singled-out components

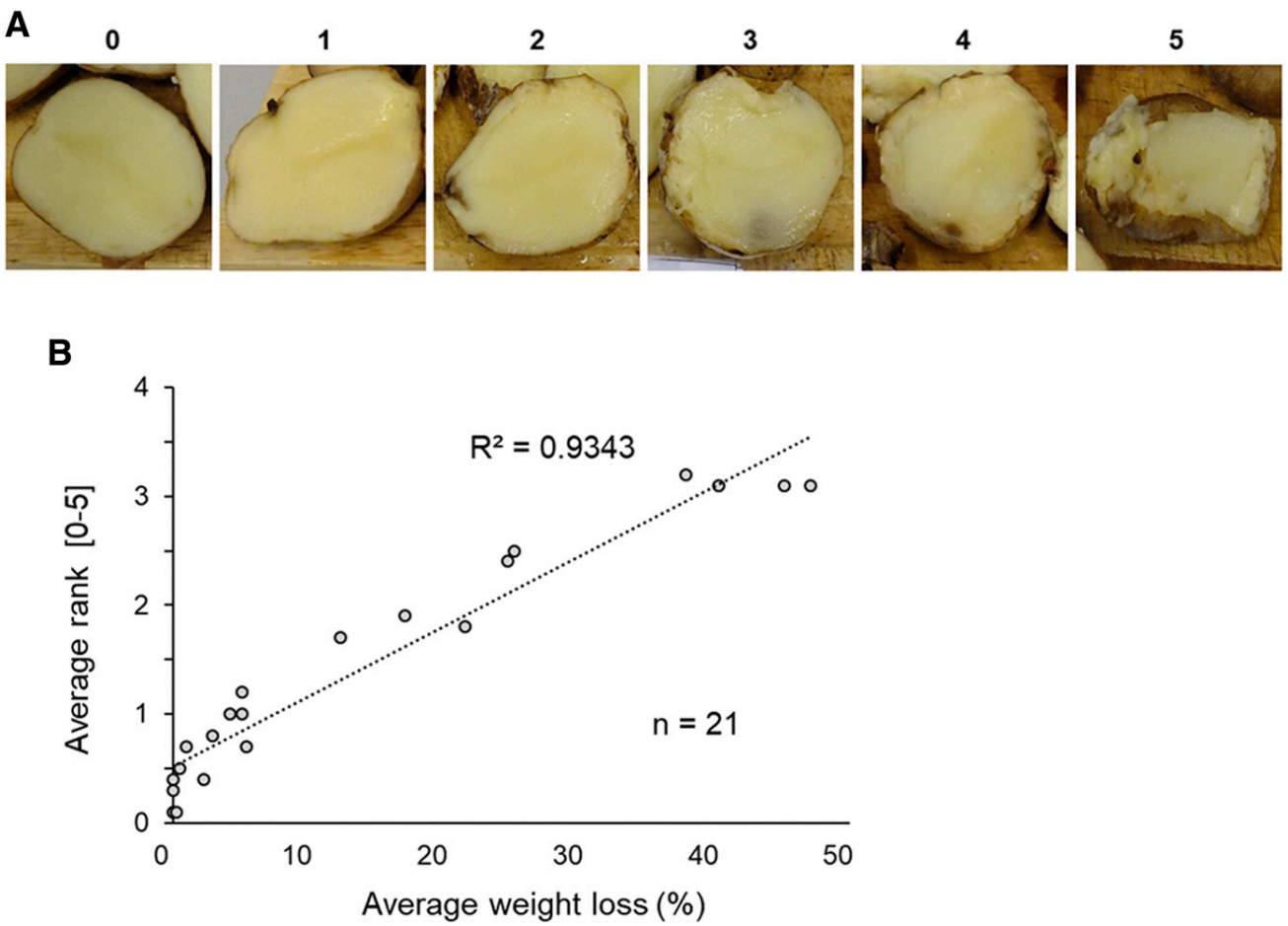

Fig. 1. Assessment of soft rot development in potato tubers. (**A**) Six-rank disease severity scale developed for the evaluation of soft rot symptoms on vacuum infiltrated potato tubers. 0 – no symptoms, 1 – rotting localized only superficially (at the periderm) and on less than 25% of tuber surface, 2 – symptoms present on 25 to 50% of tuber surface, 3 – symptoms present on 50 to 90% of the tuber, with additional detachment of the periderm from the core, 4 – symptoms present on >90% of the tuber surface and/or reaching the tuber core, 5 – whole tuber macerated. The figure shows representative photos of soft rot affected tubers graded in the adopted scale. (**B**) Correlation of disease severity ranks in the established six-rank disease severity scale with the average potato tuber weight loss due to soft rot. Correlation was calculated for certified potato tubers cv. Irga inoculated with a mixture of pectolytic pathogens alone. Analyzed data points ($n = 21$) correspond to average rank vs. average tuber weight loss obtained for 21 boxes, 10 potatoes each.

were tested, in a potato slice assay, for the ability to reduce tuber tissue decay caused by a mixture of five SRP pathogens. Rotting symptoms were reduced by 100% in comparison with control (tuber slices inoculated with pathogens alone) for all three mixtures M2, M4, and M14 and for five of the 11 individual strains *viz*. A294, A167, H145, H440, and H469 (Fig. 2). These five effective antagonists, as well as the newly composed mixture designated GF (*the Great Five*) and containing the equal ratios of each of the five antagonists (A294, A167, H145, H440, and H469), were further tested for the protection effect against the mixture of five *Pectobacterium* spp. and *Dickeya* spp. strains in a tuber injection assay. In this experimental setup, the individual strains as well as their mixture (GF) were able to reduce tuber tissue maceration by at least 50% in comparison with the control inoculated with pathogens alone (Fig. 3).

Evaluation of the developed GF mixture for protection effect against SRP in vacuum-infiltrated potato tubers. The replicated experiments on certified pathogen-free potato tubers cv. Irga (n = 120) vacuum-infiltrated with antagonistic strains and subsequently, the day after, with soft rot pathogens, were conducted to assess the protection effect of the newly developed GF mixture against a combination of five soft rot pathogens. Under disease-conducive conditions (temperature of 28°C, 85–90% relative humidity, 5 days of incubation), the GF mixture significantly reduced the severity of soft rot symptoms when compared with tubers inoculated with pathogens alone: the average rank in the symptom severity scale was reduced by 46%, and average disease incidence was reduced by 56%. The overall disease incidence for 120 tubers inoculated with pathogens alone was 38% (Fig. 4).

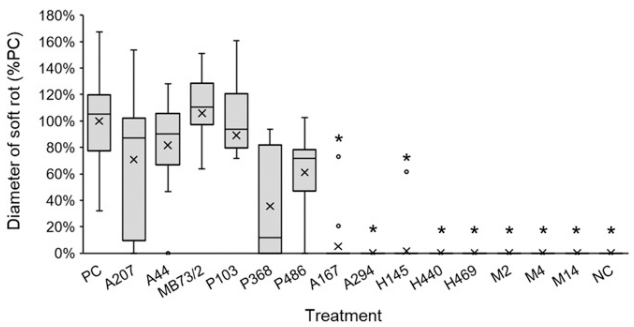

Fig. 2. Protective efficacy of mixtures M2, M4, and M14 and their singled-out components against a blend of SRP pathogens on potato tuber slices. PC – positive control slices inoculated with SRPs alone; M2, M4, and M14 – tubers co-inoculated with the respective mixtures and SRPs; NC – negative control. In the box plot, boxes determine the inter-quartile range (Q1–Q3), broad lines indicate median values, the X's are average values, whiskers indicate extreme values within 1.5 times distance from the inter-quartile range, and single data points are outliners. Each bar was created for 18 values normalized to PC. Values significantly different from PC ($P < 0.05$) are marked with an asterisk.

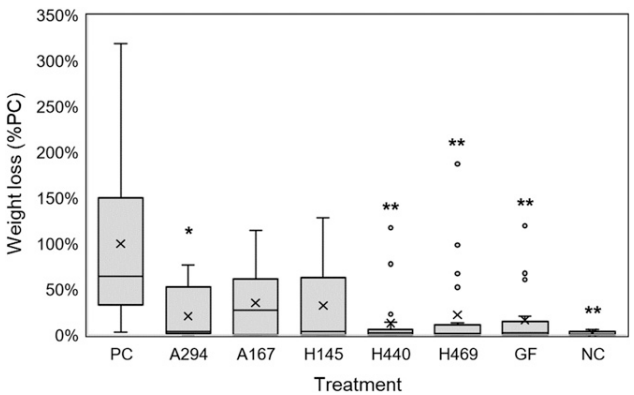

Fig. 3. Reduction of tuber weight loss to soft rot by the application of antagonists in a whole tuber injection assay. PC – positive control: tubers inoculated with a mixture of SRP pathogens alone; A294, A167, H145, H440, H469 – tubers co-inoculated with the respective antagonists (single strains) and the mixture of SRPs; GF – tubers inoculated with the GF mixture of antagonists and the SRPs; NC – negative control. Data were normalized to the average for positive control. Results from two independent experiments were pooled for analysis (n = 20). In the plot, boxes determine the inter-quartile range (Q1–Q3), broad lines indicate median values, the X's are average values, whiskers indicate extreme values within 1.5 times distance from the inter-quartile range, and single data points are outliners. Values indicated by asterisks are significantly different from PC: * $P < 0.05$; ** $P < 0.01$.

Density effect of the GF mixture on tuber rotting caused by pectinolytic bacteria. The effect of the inoculum density of the GF mixture of antagonistic bacteria on its ability to protect potato tuber tissue against soft rot caused by pectinolytic bacteria when co-inoculated *via* vacuum infiltration on potato tubers was tested. Maceration of tuber tissue by pectinolytic bacteria was inhibited by GF at the density of 10^8 CFU ml^{-1} and 5×10^8 CFU ml^{-1}, but no significant protection was observed for 10^7 CFU ml^{-1} (data not shown).

Allocation of antagonists comprising the GF mixture to species and risk categories. The five selected antagonists were assigned to particular species based on their 16S rDNA gene sequences (>1,400 bp). The classification of strains is as follows: A294 – *Serratia plymuthica*, A167 – *Enterobacter amnigenus*, H145 – *Rahnella aquatilis*, H440 – *Serratia rubidaea*, and H469 – *Serratia rubidaea*. Sequence identity between the 16S rDNA genes of the studied strains and the reference sequences of the respective species available in the GenBank database was 99 to 100% (data not shown). All five selected antagonists are conceded as GRAS (Generally Recognized As Safe) according to the American Food and Drug Administration (FDA). They all belong to the risk category 1 according to ATCC; they are neither known to consistently cause disease in healthy individuals nor do they pose risk for animals, plants, and the environment.

Growth of selected antagonists at different temperatures. In replicated experiments, growth of A294, A167, H145, H440, and H469 strains was determined at 28, 37, 40, and 42°C in liquid medium over a period of 16 h and at 7, 10, 28, 37, 40, and 42°C on a solid medium for 120 h. In liquid medium, all five strains grew at all tested temperatures, reaching, after 16 h, an average log CFU between 7.5 and 9.2 (Supplementary Fig. S1). On the solid medium, after 5 days of incubation, all five strains showed growth at 7, 10, 28, and 37°C. Additionally, strains A167, H440, and H469 grew at 40°C, and strains H440 and H469 grew at 42°C (Supplementary Table S3).

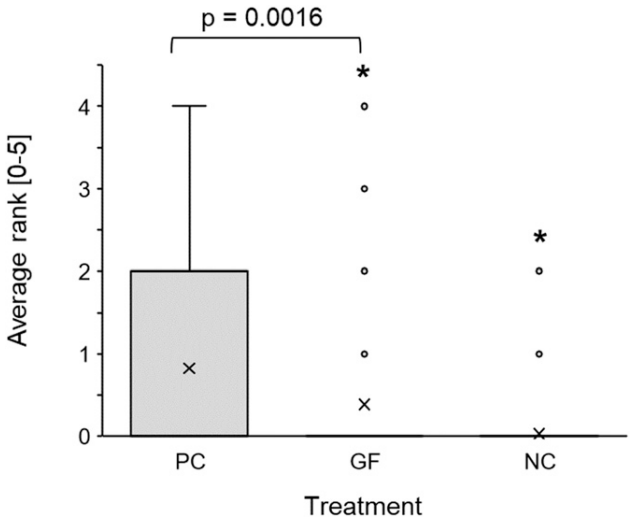

Fig. 4. Efficacy of the GF mixture of antagonists in suppressing soft rot symptoms caused by a blend of SRP pathogens on vacuum-infiltrated potato tubers. Box plot shows the disease severity ranks obtained for tubers infiltrated with: PC – tubers infiltrated with SRP pathogens alone (positive control); GF – tubers infiltrated subsequently with the GF mixture of antagonistic strains and the SRP pathogens; NC – tubers infiltrated with water (negative control). Values shown in the graph were derived from 4 independent experiments, 30 tubers each (n = 120). Confidence level for difference between the positive control and the GF treatment is given in the graph.

Antibiotic susceptibility profile of the selected antagonistic strains. Strains A294, A167, H145, H440, and H469 were evaluated for their susceptibility to 21 commercially available antibiotics. All strains were susceptible to: chloramphenicol doxycycline, colistine, piperacillin + tazobactam, aztreonam, imipenem, ciprofloxacin, tigecycline, gentamicin, ceftazidme, fosfomycin, rifampin, ampicillin + sulbactam, and streptomycin and were resistant to: oxacillin, dalfopristin + quinupristin (Synercid), clindamycin, linezoil, vancomycin, and fusidic acid. Strain H145 was resistant to ampicillin, unlike strains A294, A167, H440, and H469. In total, strains A294, A167, H440, and H469 showed susceptibility to 15 antibiotics, and strain H145 was susceptible to 14 of the 21 antibiotics tested (Supplementary Table S2).

Influence of the selected antagonists on the survival of *Caenorhabditis elegans*. At the end point of the liquid killing assay (3 days), the average survival rate of *C. elegans* cultivated on *E. coli* OP50 as a food source (negative control) was 91%. At the same time, no viable nematodes were found in the presence of *P. aeruginosa* PA14, known for its killing potential to the nematode (positive control). The survival rate for *C. elegans* treated with either A294, A167, H145, H440, or H469 ranged from 58 to 91%. Among the tested strains, A294 and H440 ensured the lowest average survival rates (58 and 65%, respectively). Nematode survival rates obtained for treatments containing A167, H145, and H469 amounted to 88, 90, and 81%, respectively, and were not significantly statistically different from the negative control (OP50) (Supplementary Fig. S2).

Discussion

This study was conducted to develop and evaluate a mixture of antagonistic bacterial strains for protection of potato seed tubers against SRP. Numerous bacterial antagonists against different *Pectobacterium* and *Dickeya* species have been isolated and characterized previously to control these bacteria on potato and other crops (Diallo et al. 2011). However, this study, to our knowledge, is the first dealing with a stepwise screening to develop a mixture of antagonists to be used against a spectrum of different *Pectobacterium* spp. and *Dickeya* spp. applied simultaneously, and under high disease pressure (Boyd 1972). This approach was taken to simulate a worst-case scenario in which a susceptible host under adverse environmental conditions is challenged simultaneously by several SRP pathogens present in high numbers. Therefore, we postulated that if the mixture of antagonists is able to provide protection against SRP under the proposed experimental conditions, it will be also able to control the bacteria under natural conditions less favorable to disease development (Pérombelon and Lowe 1975).

To date, no biological control measures exist to protect potato tubers against SRP bacteria. The use of compatible mixtures of antagonistic bacteria has, however, many advantages over applications containing single biological control agents (Rechcigl 2017). Application of such artificial communities may provide better protection of the plant thanks to a broader range of pathogen-suppressive mechanisms, more efficient colonization of the host, and higher persistence in the plant environment, i.e., due to higher adaptation to changes throughout the growing season. Mixtures are also more likely to be effective against a wider range of pathogens at the time (Siddiqui and Shaukat 2002; Stockwell et al. 2010).

Although for the initial screening we used 22 antagonists well-characterized in our previous studies, distributed randomly in 15 mixtures of 4 to 5 strains each, only three of these mixtures: M2, M4, and M14 protected potato tubers from rotting caused by a combination of five *Pectobacterium* spp. and *Dickeya* spp. This can be partly explained by the fact that the individual antagonists were selected initially against a limited number of SRP strains (Jafra et al. 2006, 2009; Krzyzanowska et al. 2012a, b). The biocontrol potential of some of them was reported to be highly dependent on the species of soft rot pathogen (Krzyzanowska et al. 2012b). Similarly, it cannot be excluded that some of the antagonists also inhibited other members of the mixture. The majority of strains used in this study originate from the rhizospheres of diverse plants (Table 1). These niches are highly competitive, and it is widely accepted now that in such complex environments microorganisms most often express competitive phenotypes (Foster and Bell 2012), which could affect development of new biocontrol mixtures (Jetiyanon and Kloepper 2002).

The final outcome of this study, the GF mixture of five effective antagonists, provided stable and significantly high level of seed tuber protection against all tested SRP under disease favorable conditions. The bacteria present in this mixture possess different mechanisms by which they can control SRP on potato tubers. This includes direct antagonism *via* production of antibiotic compounds and biosurfactants (strains A294, H145, H469), inactivation of signal molecules regulating expression of virulence factors in SRP (so-called quorum quenching) (strains A167, H440), and production of siderophores chelating iron ions in environment (strains H145, A294, H440, H469) (Table 1). These features have all been reported to play a role in biological control of phytopathogens including potato-infecting SRP (Baker 1987; Shoda 2000). Mixtures of bacterial antagonists can be applied as stand-alone plant protection measures, as demonstrated in this study, making them of interest in organic crop production. It is equally possible to apply these agents together with conventional treatments to reduce the use of chemicals and/or to improve their efficiency in agricultural applications (Ferron and Deguine 2005).

We consider that *S. plymuthica* strain A294, *E. amnigenus* strain A167, *R. aquatilis* strain H145, *S. rubidaea* strain H440, and *S. rubidaea* strain H469 are potentially good candidates for developing a commercial protection product for potato tubers for several reasons. In our assays, each of the strains alone provided a significant protection of tubers against rotting caused by SRP under disease promoting conditions and in different setups. Furthermore, in former studies, three of the strains comprising the GF mixture, namely H145, H440, and H469, were shown to have a protective effect against soft rot of hyacinth bulbs (Jafra et al. 2009). This implies that the developed mixture is likely to be applicable on other soft rot-affected agricultural plants and ornamentals. Likewise, the five strains are all classified into risk category 1 according to ATCC and recognized as GRAS according to FDA, meaning that they are not expected to pose risks for humans and/or the environment. As well, they are susceptible to the commercially available antibiotics. Members of these species have been already used in (commercial) biological control applications on different crops including strawberry, cucumber, oilseed rape, potato, grape, tomato, apple, and others (Calvo et al. 2007; Chen et al. 2007; De Vleesschauwer and Höfte 2003; El-Hendawy et al. 2005). Furthermore, in experiments in which *C. elegans* was grown on A294, H145, A167, H440, or H469, all investigated strains showed significantly less pathogenicity toward nematodes compared with well-known nematode-killing *P. aeruginosa* strain PA14. In all cases, nematodes treated with the antagonistic bacteria survived till the end of experiment. *C. elegans* killing assay is a well-accepted model system to study bacterial pathogenicity toward eukaryotic organisms. This model is also recommended as a first test to assess if a particular isolate can be considered as biological control agent to be used in agriculture (Zachow et al. 2009).

We propose to use the developed screening system as a method of choice for a more reliable selection of antagonistic mixtures. Briefly, once the initial individual antagonists of choice have been selected, several random mixtures containing up to five antagonists each are prepared and tested in assays with pathogens under disease favorable conditions. Some of the tested mixtures would provide better protection than others, and these should be selected for further studies. In the next step, the individual antagonists from the selected mixtures are then tested alone using either a simpler laboratory assay or the same assay as the one used for the initial screening. The antagonists providing the best protection should be selected and finally mixed together to create a new working composition which is evaluated under disease favorable conditions (Supplementary Fig. S3). This step-wise selection of antagonists' mixture could be applied commonly and work not only on potato but also on other crop systems.

In conclusion, although the results obtained in this study are promising for biocontrol of soft rot caused by a mixture of SRP and under conditions promoting disease development, there is still considerable work to be done to achieve a viable commercial application. Points

that require further examination are: formulation of a bacterial preparation, optimization of application procedures, assessing the longevity of the applied mixture on (seed) potato tubers during storage and in transit, as well as the effectiveness of the mixture when applied on a wide range of potato cultivars and under different storage conditions. Finally, elucidation of the molecular basis of interactions between the five antagonistic strains in the mixture will be of interest, not only to contribute to fundamental knowledge, but also to explore the use of this combination in other pathogen–host systems.

Author Contributions

Conceptualization: DK, TM, RC; data curation: DK, TM; formal analysis: DK, SJ, RC; funding acquisition: RC, MK; investigation: DK, TM, JS, MK; methodology: DK, TM, MK, JS; project administration: DK, RC; resources: RC, MK; supervision, validation: SJ, RC; visualization: DK, TM, MK, RC; writing ± original draft: DK, RC; writing ± review and editing: DK, SJ, RC

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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. J. Syst. Evol. Microbiol. 55:1415-1427.

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2.2. The Great Five—an artificial bacterial consortium with antagonistic activity towards *Pectobacterium* spp. and *Dickeya* spp.: formulation, shelf life, and the ability to prevent soft rot of potato in storage [1]

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Short description

This manuscript describes developing the formulation of the previously described consortium of antagonistic bacteria. This is essential in developing biocontrol products that are often overlooked (Stephens & Rask, 2000). Furthermore, we present the shelf life of the formulated bacteria and results from on formulated bacteria against Soft Rot Disease in the target setup. Evaluating these critical features is necessary to confirm if a given biocontrol agent can be potentially used in agriculture (Bashan et al., 2014).

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ENVIRONMENTAL BIOTECHNOLOGY

The Great Five—an artificial bacterial consortium with antagonistic activity towards *Pectobacterium* spp. and *Dickeya* spp.: formulation, shelf life, and the ability to prevent soft rot of potato in storage

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Abstract

"The Great Five" (GF) is an artificial bacterial consortium developed to protect potato tubers from soft rot caused by *Pectobacterium* spp. and *Dickeya* spp. To investigate the commercialization potential of the GF, we developed liquid and powder formulations of the consortium and of each of the comprising strains (*Serratia plymuthica* strain A294, *Enterobacter amnigenus* strain A167, *Rahnella aquatilis* strain H145, *Serratia rubidaea* strain H440, and *S. rubidaea* strain H469). To form powders, the cells were lyophilized using a newly developed lyoprotectant: Reagent PS. The shelf life of the formulations stored at 8 and 22 °C was monitored for a period of 12 months. The longest shelf life was obtained for formulations stored at 8 °C; however, the viability of all formulations was negatively affected at 22 °C. For the consortium, a 2.5 $\log_{10}$ cfu (colony forming units) drop in cell number was recorded for the liquid formulation after 6 months, while in case of powders, the drop remained below 1 $\log_{10}$ cfu following 12 months. The ability of the powder formulations to preserve biocontrol activity of the consortium was tested on potato tubers treated with the formulations and a mixture of the soft rot pathogens. The inoculated tubers were stored for 6 months at 8 °C to mimic commercial storage conditions. Soft rot severity and incidence on potato tubers treated with formulations were significantly reduced (62–75% and 48–61%, respectively) in comparison to positive control with pathogens alone. The potential use of the newly developed formulations of "The Great Five" for the biocontrol of soft rot is discussed.

Key Points

- *An innovative reagent to protect bacterial cells during lyophilization was developed.*
- *Powder formulations of "The Great Five" prolonged its shelf life.*
- *The powder-formulated "The Great Five" was active against soft rot bacteria on potato tubers.*

Keywords Pectinolytic *Erwinia* · Blackleg · Biological control · Antagonism · Potato

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Introduction

Pectinolytic Soft Rot *Pectobacteriaceae* (SRP: *Pectobacterium* spp. and *Dickeya* spp.; former pectinolytic *Erwinia* spp.) infect a number of plant species worldwide including agriculturally relevant crops (Toth et al. 2003). SRP are recognized among the top 10 most important bacterial pathogens in agriculture (Mansfield et al. 2012). In potato, these pathogens cause a variety of disease symptoms including pre-emergence decay of tubers, aerial stem rot, and blackleg under field conditions, as well as soft rot of progeny tubers in storage (Pérombelon 2002). In Europe, the high losses in (seed) potato production are predominantly associated with declassification and rejection of lots. This includes

reduction of the market value due to the infestation with SRP (Toth et al. 2011).

It is widely accepted that the major source of SRP in the environment are latently infected potato tubers (Pérombelon 1974). Latently infected tubers can carry a relatively high SRP inoculum reaching after storage period even 10^2–10^4 viable cell per gram of tuber tissue. This inoculum is enough to cause soft rot symptoms in the next growing season (Czajkowski et al. 2009). Latent infections promote transmission of the inoculum as the pathogens may spread unnoticed through several genera-tions of tubers before the occurrence of disease symptoms (Pérombelon 1992). Consequently, the production of pathogen-free seed material and/or application of protective measures against contamination with *Pectobacterium* spp. and *Dickeya* spp. remain of utmost importance (Pérombelon 1992).

A well-recognized method to reduce tuber contamination with SRP is the use of pathogen-free seed material derived from axenic potato cultures (Gopal et al. 1998). However, the use of SRP-free planting material at startup does not prevent infections that may occur later during potato cultivation in the field (Pérombelon 1974). Apart from the infected potato plants in the field and/or soft rotting tubers in storage that spread inoculum to healthy tubers, the potential reservoirs of the pectinolytic bacteria include common weeds (Tsror et al. 2010) and non-host plants (Fikowicz-Krosko et al. 2017; Toth et al. 2011), surface water (Cappaert and Powelson 1987; Harrison et al. 1987; McCarter-Zorner et al. 1984), and soil (Toth et al. 2003). Because the pathogen inoculum required to establish an infection in potato is low (Toth et al. 2003), the initially SRP-free seed tubers can rapidly become symptomatic following planting in an "open environment" (Charkowski 2015; Toth et al. 2011).

Aside from hygiene measures to prevent contamination of plant material with SRP (Czajkowski et al. 2011), potato tuber treatments may be recognized as an additive approach to increase the quality of the potato (seed) lots. Physical and/or chemical tuber treatments developed for pathogen control measures were reported to provide some level of control of infections caused by *Pectobacterium* spp. and *Dickeya* spp. in potato (Mills et al. 2006; Ranganna et al. 1997). Many of these treatments, however, show considerable phytotoxicity to the treated tubers, therefore decreasing their viability, and are difficult to apply on a large scale in the potato production systems (Pérombelon 1992), or are unable to target SRP cells localized deep in the vascular tissues (Czajkowski et al. 2011).

An environmentally friendly alternative to chemical and/or physical tuber treatments is biological control. Microbial biocontrol agents reduce the population size of the pathogens and/or suppress their virulence using different antagonistic mechanisms (Compant et al. 2005). Several publications describe the isolation and characterization of bacterial antagonists effectively controlling potato tubers against *Pectobacterium* spp. and *Dickeya* spp. (Czajkowski et al. 2012; De Essarts et al. 2016; Jafra et al. 2009; Krzyzanowska et al. 2019b, 2012b). However, none of the biocontrol agents reported so far have been used as commercial products against SRP (Czajkowski et al. 2011).

One of the major challenges in commercial application of microbial biocontrol agents is the formulation of the working microbial inoculum (Stephens and Rask 2000). Successful formulations must enable biological control agents to remain viable during long-term storage and then become metabolically active in the environment upon application (Bashan et al. 2014). Choosing an efficient method to formulate a biocontrol strain is not trivial. Due to the variety of microbial species with different biocontrol activities, no universally applicable method exists to preserve the viability of bacterial strains used in biocontrol products (Stephens and Rask 2000). Furthermore, the final product must be compatible with the requirements of the target cropping system, safe, and relatively inexpensive to use (Bashan et al. 2014; Fravel 2005).

Microbial formulations can be prepared as solids or liquids (Berninger et al. 2018). Solid-state formulations include powders and granules, whereas liquid formulations comprise microbial cells suspended in water-based or oil-based carriers (Stephens and Rask 2000). Each of the formulation types offers different benefits. For example, granules containing encapsulated bacteria enable stable release of the microorganisms to the environment and are therefore advantageous for soil applications where timed release of the antagonists is critical (Bashan 1986). Powder formulations containing desiccated bacteria usually offer the best shelf life of the formulated inoculum (Meng et al. 2008). Finally, liquid formulations, although not as stable as solids and, in general, offering shorter shelf life of the inoculum, are the most straightforward to prepare, they are readily compatible with most agricultural equipment and are fit also for foliar application (Bashan et al. 2014).

Recently, we developed and described a (artificial) consortium of five antagonistic bacterial strains, designated the Great Five (GF), comprising: *Serratia plymuthica* strain A294, *Enterobacter amnigenus* strain A167, *Rahnella aquatilis* strain H145, *Serratia rubidaea* strain H440, and *S. rubidaea* strain H469. The GF consortium has been developed in our former study after several rounds of experiments in which consortia containing random selection of antagonistic bacterial strains were created and evaluated for protection of potato tubers against SRP. After each round, the best candidates were selected from the starting consortia and finally combined into the new GF consortium, which has been then extensively evaluated again against a mixture of SRP on potato tubers under disease-favorable conditions and with high pathogen load (Krzyzanowska et al. 2019a, b).

The GF consortium efficiently protects potato tubers from soft rot caused by a combination of SRP pathogens and under conditions promoting disease development (Krzyzanowska et al. 2019a, b). In experiments where inoculated tubers were treated with suspension of freshly grown bacterial cells,

followed by immediate transfer to disease-favoring conditions (high temperature, high humidity), the GF consortium reduced soft rot incidence by as much as 46% in comparison with the control, which comprises tubers inoculated with a mixture of SRP pathogens.

In this study, we aimed to develop a formulation of the GF consortium that could be applied to the surface of potato tubers prior to storage and/or before planting to protect them against SRP. A set of different formulations was prepared and evaluated in terms of preserving bacterial viability (shelf life of the formulated bacterial strains) at two different temperatures, 8 and 22 °C, for a total period of 12 months. The formulations were also tested for the ability to suppress soft rot symptoms on treated tubers following 6-month storage in a cold room (8 °C). Moreover, as the preparation of the solid powder formulations required desiccation of cells with minimal loss of viability, we developed an innovative lyoprotectant and evaluated its efficacy in preserving cells during freeze drying. The results of the study and their implications for the biocontrol of SRP in potato with artificial (and formulated) microbial consortia are discussed.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. The SRP pathogens and the biological control strains of the Great Five consortium (GF): *S. plymuthica* strain A294 (Polish Collection of Microorganisms, Wroclaw, Poland (PCM) B/00143), *E. amnigenus* strain A167 (PCM B/00145), *R. aquatilis* strain H145 (PCM B/00144), *S. rubidaea* strain H440 (PCM B/00141), and *S. rubidaea* strain H469 (PCM B/00142) were grown for 24–48 h at 28 °C on Tryptone Soy Agar (TSA; Oxoid, Basingstoke, UK) or in TSB. Liquid bacterial cultures were agitated (150 rpm) during cultivation. The probiotic microorganisms (*Bacillus coagulans* (Colinox), *Lactobacillus brevis* 269Y, *Lactobacillus rhamnosus* GG, *L. rhamnosus* 573, and yeast *Saccharomyces boulardi* (ENTEROL 250)), were grown at 37 °C in De Man, Rogosa, and Sharpe medium (MRS, BTL Ltd., Warsaw, Poland), and *Eschericha coli* strain DH5α, grown in Tryptone Soy Broth (TSB; Oxoid, Basingstoke, UK) for 24–48 h at 37 °C.

Freeze drying (lyophilization) of bacterial cells

Small-scale lyophilization (up to 80 mg fresh weight) was performed to evaluate how the studied biological control strains comprising the GF consortium of antagonists (A294, A167, H145, H440, and H469) survive the freeze-drying procedure in the presence and absence of lyoprotectants. To provide a point of reference, the experiment was additionally carried out on 10 other microorganisms: three plant-associated bacterial strains (*Ochrobactrum quorumnocens* A44 (Krzyzanowska et al. 2019a, b), *Pseudomonas donghuensis* P482 (Krzyzanowska et al. 2012a), and *Pseudomonas protegens* CHA0 (Stutz et al. 1986), five probiotic microorganisms (*B. coagulans*, *L. brevis* 269Y, *L. rhamnosus* GG, *L. rhamnosus* 573, and yeast *S. boulardi*), and two other well-studied model bacterial strains: *Bacillus subtilis* 168 and *E. coli* DH5α (Tab.1). The two tested lyoprotectants included Reagent 18, recommended for freeze drying of microorganisms by the American Type Culture Collection (ATCC) (per 100 mL: 0.75 g TSB, 10 g sucrose, 5 g bovine serum albumin (BSA)), and Reagent PS—a modified version of Reagent 18 in which a bovine serum albumin (BSA) was replaced with a wheat peptone (per 100 mL: 0.75 g TSB, 10 g sucrose, 0.255 g wheat peptone (Sigma-Aldrich, Darmstadt, Germany)(Polish patent application P.428215, 2018).

For small-scale lyophilization, cells from 1 mL of overnight bacterial cultures were pelleted (4500×g, 5 min) and weighted. Each 250 mg of cell fresh weight (fw) was re-suspended in 1 mL of either Reagent 18, Reagent PS, or sterile distilled water (negative control). Cell suspensions representing all 45 combinations (15 strains, each in 3 lyophilization media) and in three technical replicates each (n = 135) were frozen overnight at − 80 °C and subsequently freeze dried for 24 h at − 50 °C in the Heto PowerDry Freeze Dryer (Thermo Scientific, Warsaw, Poland). The dry pellets were thoroughly re-suspended in sterile distilled water, in a volume equal to the initial volume of the sample (1 mL). The number of viable cells was determined before and after freeze drying by plating 10 μl 10-fold serial dilutions of bacterial suspen-sions on a suitable growth medium—MRS agar for probiotic microorganisms and TSA for all other strains. Each dilution was plated in three technical replicates. Per strain and lyoph-ilization medium, cell survival rate was calculated according to the equation:

$$\text{survival rate}\ (\%) = \frac{\text{cfu g}^{-1}\text{fw after freeze drying}}{\text{cfu g}^{-1}\ \text{fw before freeze drying}} \times 100\%$$

where cfu—colony forming units; fw—cell fresh weight (Miyamoto-Shinohara et al. 2006).

Large-scale lyophilization (up to 80 g fresh weight) of the GF strains A294, A167, H145, H440, and H469 was outsourced to Pomeranian Science and Technology Park in Gdynia (PPNT, Gdynia, Poland). As a part of this service, the bacterial isolates were individually cultured in 10 L of TSB and freeze dried in 100 mL of Reagent PS per 25 g of bacterial fresh weight (fw). For each strain, the procedure was performed twice, yielding two independent batches of freeze-

Table 1 Bacterial strains used in this study

Species	Strain	International culture collection no.	Reference/source
Strains of the GF consortium of antagonists			
Enterobacter amnigenus	A167	PCM B/00145	(Jafra et al. 2006)
Rahnella aquatilis	H145	PCM B/00144	(Jafra et al. 2009)
Serratia plymuthica	A294	PCM B/00143	(Jafra et al. 2006)
Serratia rubidaea	H440	PCM B/00141	(Jafra et al. 2009)
Serratia rubidaea	H469	PCM B/00142	(Jafra et al. 2009)
SRP bacteria comprising the mix of five plant pathogens			
Pectobacterium atrosepticum	SCRI 1043	ATCC BAA-672	(Bell et al. 2004)
Pectobacterium carotovorum subsp. *carotovorum*	Ecc71	ATCC 15713	(Willis et al. 1987)
Pectobacterium parmentieri	SCC3193	CFBP 8475, LMG 29774, LMG:29774	(Pirhonen et al. 1991)
Dickeya solani	IPO2222	DSM 28711, LMG 25993, NCPPB 4479	(van der Wolf et al. 2014)
Dickeya dianthicola	CFBP 1200	NCPPB 453 T, ICMP 6427 T LMG 2485 T	(Samson et al. 2005)
Probiotic microorganisms supplemented to humans			
Bacillus coagulans	Not indicated	Not indicated	Colinox (VITAMED)
Lactobacillus brevis	269Y	DSM 20556, ATCC 8287	(Davis 1955)
Lactobacillus rhamnosus	GG	ATCC 53103	(Goldin et al. 1992)
Lactobacillus rhamnosus	573	Not indicated	Lactovaginal (IBSS Biomed)
Saccharomyces boulardi (yeast)	not indicated	Not indicated	ENTEROL 250 (BIOCODEX)
Plant-associated bacteria			
Ochrobactrum quorumnocens	A44	LMG 30544 PCM 2957	(Krzyzanowska et al. 2019a, b)
Pseudomonas donghuensis	P482	CCTCC AB 2012141 NRRL B-59108	(Krzyzanowska et al. 2012a)
Pseudomonas protegens	CHA0	DSM 19095 LMG 27888	(Stutz et al. 1986)
Other			
Bacillus subtilis	168	BGSC 1A700	(Ehrenberg 1834)
Escherichia coli	DH5α	NCTC 13450	(Anthony and Bailey 1989)

GF the Great Five; a consortium of bacterial antagonists (A294, A167, H145, H440, H469) shown to attenuate soft rot caused by the SRP pathogens (Krzyzanowska et al. 2019a, b *PCM*, Polish Collection of Microorganisms; Wroclaw, Poland, https://www.pcm.org.pl/home, patent deposit according to the Budapest treaty; *SRP*, Soft Rot *Pectobacteriaceae*, plant pathogenic bacteria of genera *Dickeya* and *Pectobacterium*, causing soft rot and blackleg diseases on vegetables and ornamental plants

dried cells. The lyophilizates were stored in glass jars in the presence of silica gel desiccant, at 8 °C in the dark.

Formulation

Each strain of the GF consortium (A294, A167, H145, H440, and H469) was formulated into two wettable powders (WPs), one liquid preparation (LQ), and a control (CTRL). The WPs comprised of bacterial lyophilizate, a carrier: kaolinite (ZielonyKlub.pl, Poland) or diatomaceous earth (Perma-Guard, Otwock, Poland), and a common mix of chemicals reported in literature to increase shelf life of bacterial formulations (Arora and Mishra 2016), that is: methyl cellulose 15 cP (mPa s) (Sigma-Aldrich, Darmstadt, Germany), cyclodextrin (Sigma-Aldrich, Darmstadt, Germany), sodium lignosulphonate (Roth, Karlsruhe, Germany, and KH_2PO_4 (POCH, Warsaw, Poland). The wettable powder (WP) with the kaolinite carrier was designated WP-KAO, and the WP with diatomaceous earth carrier was designated WP-DE. The composition (in %) of all formulations is given in Table 2.

To prepare the liquid formulation, designated LQ cells were freshly cultured overnight on TSA at 28 °C, harvested by scraping them from the agar, and suspended in 1/4 Ringer's buffer (Merck, Darmstadt, Germany) to obtain the turbidity of

Table 2 Composition of the tested bacterial formulations

Component[a]	Liquid		Powder (dry)		
	CTRL	LQ	LYO	WP-KAO	WP-DE
Mix of GF strains[b]	100% (suspension)	89.9% (suspension)	100% (lyophilizate)	60% (lyophilizate)	60% (lyophilizate)
Solid carrier[c]	–	–	–	29.9%	29.9%
Methyl cellulose 15 cP	–	5%	–	5%	5%
Cyclodextrin	–	0.1%	–	0.1%	0.1%
Sodium lignosulphorate	–	4%	–	4%	4%
KH_2PO_4	–	1%	–	1%	1%

[a] The percentages are given in or w/v ratios for LQ and in w/w ratios for WP-KAO and WP-DE

[b] Bacterial strains were mixed in equal v/v or w/w ratios. In all combinations, the total titer of bacterial cells in the final formulation/control equaled ca. 5×10^{10} cfu mL^{-1} in the liquid suspensions (CTRL, LQ) and 1×10^{11} cfu g^{-1} in powders (LYO, WP-KAO, WP-DE). Cell suspensions were prepared in 1/4 Ringer's buffer and the lyophilizates were obtained using the PS lyoprotectant

[c] In case of WP-KAO, the solid carrier was kaolinite. In case of WP-DE, the carrier was diatomaceous earth; "–" component not added to a given consortium

15 McF (ca. 5×10^{10} cfu mL^{-1}). The suspension was supplemented with 100 mg mL^{-1} of LQ formulation mix (49.5% methyl cellulose 15 cP, 0.99% cyclodextrin, 39.6% sodium lignosulphonate, 9.9% KH_2PO_4). The 100 mg of this formulation mix was added per 1 mL of bacterial suspension which resulted in a final concentration of the additives analogous to that applied for the WPs (Table 2). Fresh bacterial suspensions in 1/4 Ringer's buffer but without the formulation mix were used as control (CTRL).

To reliably determine the shelf life of the formulations, defined as the viability of bacteria over time (Berninger et al. 2018), two individual lots were prepared for each formulation, with a postponement between them of 8 months. For powder formulations, each lot comprised of cells freeze-dried in inde-pendent procedures.

Viability of the antagonistic strains of GF during long-term storage

The shelf life of individual strains: A294, A167, H145, H440, H469, and their mixture, the GF consortium was investigated in two liquid and three powder preparations. The liquid prep-arations included the LQ formulation and the control suspen-sions in 1/4 Ringer's buffer (CTRL). The powder preparations included the lyophilizate (LYO) without the addition of for-mulation mix and two lyophilizate-based WPs: WP-KAO and WP-DE.

To store powder formulations for the shelf life experiment, the formulations (LYO, WP-KAO, or WP-DE) were placed in a sterile 5-mL Eppendorf tube. The samples, each in two tech-nical replicates, placed in a plastic box with silica gel desiccant were stored at either 8 or 22 °C for a total period of 12 months.

To store liquid preparations (LQ and CTRL), 5 mL of each sample, two technical replicates each, were aliquoted into sterile wide-mouth amber glass bottles with stoppers (Bionovo, Legnica, Poland). The bottles were kept at either 8 or 22 °C for 12 months.

All formulations (LYO, WP-KAO, WP-DE, LQ) and the control (CTRL) were sampled immediately after preparation and, subsequently, following 1, 2, 3, 6, 9, and 12 months of storage at both temperatures (8 and 22 °C). At the respective time points, aliquots of the powder formulations (LYO, WP-KAO, and WP-DE) (ca. 100 mg) were collected with a sterile spatula, weighed, and thoroughly re-suspended in 1 mL of sterile distilled water per each 100 mg of the powder. Aliquots of 100 μL were collected in case of the liquid preparations (LQ and CTRL). The viability of the cells in each preparation was determined by dilution plating on TSA as described above for the assessment of the efficiency of lyoprotectants. Results were expressed in cfu g^{-1} for the dry and in cfu mL^{-1} for the liquid preparations. In order to compare the shelf life of different formulations, slope of the survival curve (x), expressing the average decline in $\log_{10}$ cfu per month of storage, was calculated with the following equation:

$$x = \frac{\sum(t_i - \overline{t})(y_i - \overline{y})}{\sum(t_i - \overline{t})^2}$$

where y_i stands for the count of viable cells ($\log_{10}$ cfu g^{-1} or $\log_{10}$ cfu mL^{-1}) at the corresponding time of sampling (t_i, in months), y is the average of all y_i values, and t is the average of all t_i values. The lower the slope value, the steeper decrease in the number of viable cells was observed over time. For storage at 8 °C, the whole experiment was performed twice, separately for each lot of formulations (lot 1 and lot 2). For 22 °C, due to low cell survival in the first experiment, the experiment was not repeated.

Biocontrol efficacy of bacterial preparations—protection of potato tubers against SRPs in storage

High-quality, pathogen-free seed tubers cv. Irga (caliber 30–50 mm), showing moderate resistance to soft rot (4.0 in 9.0 rank scale; http://ziemniak-bonin.pl), were purchased from a potato breeding company (Pomorsko-Mazurska Hodowla Ziemniaka, ang. Pomeranian-Masurian Potato Breeding Szyldak, Poland, http://www.pmhz.pl/). The potato tubers cv. Irga expressing moderate resistance to soft rot were chosen to mimic the natural field/storage situation. The moderate resistant cultivars are commonly used by farmers worldwide. The susceptible cultivars are not used commercially due to the high soft rot incidence and consequently high losses. Soft rot immune (fully resistant) potato cultivars do not exist on the market (Czajkowski et al. 2011).

The tubers were subsequently treated, by vacuum infiltration (Czajkowski et al. 2012) with the GF consortium of antagonists (A294, A167, H145, H440, and H469 in equal ratios) and a composition of five soft rot pathogens of genera *Pectobacterium* and *Dickeya* (*Pectobacterium atrosepticum* strain SCRI 1043, *Pectobacterium carotovorum* subsp. *carotovorum* strain Ecc71, *Pectobacterium parmentieri* strain 16 SCC3193, *Dickeya solani* strain IPO2222, and *Dickeya dianthicola* strain CFBP 1200 (IPO1741), representing species and subspecies known to most often cause soft rot and blackleg diseases in Europe (Pérombelon 2002; van der Wolf et al. 2017). Prior to the other experiments, the virulence of *Pectobacterium* spp. and *Dickeya* spp. strains used in this study was verified using potato slice assays as described in (Czajkowski et al. 2012).

The bacterization of tubers was performed according to a protocol modified from Krzyzanowska et al. (2019a, b). The modifications included an additional storage period in the cold room (8 °C) and the scale of the experiment (number of tubers processed per combination). Briefly, potato tubers were sur-face sterilized for 20 min in 5% commercial bleach (ACE, Procter and Gamble, Gdansk, Poland), washed 3 times with running tap water, and placed in 5-L beakers, 30 tubers each. Next, the tubers were immersed in the suspensions of the antagonists formulated as described above. The tested prepa-rations included the lyophilizate (LYO), two WPs (WP-KAO and WP-DE), and unpreserved cells from fresh cultures on TSA medium suspended in ¼ Ringer's buffer (FR). The met-abolically active cells as present in FR were not previously tested in a long-term storage experiment. Moreover, FR pro-vides a point of reference for the performance of formulated (preserved) cells in LYO, WP-KAO, and WP-DE. Working solutions of dry preparations were obtained by adding 500 mg of powder to 1 L of tap water (ca. 5×10^8 cfu mL^{-1} of antag-onistic bacteria in total, based on the count of viable cells in the stored formulations at the time of the experiment). The FR suspension (also 5×10^8 cfu mL^{-1} of bacteria in total), as well as the mix of five soft rot pathogens (1×10^6 cfu mL^{-1} of bacteria in total, 5×10^5 cfu mL^{-1} of each individual strain), were prepared as described in Krzyzanowska et al. (2019a, b). The immersed tubers were placed in a desiccator and vacuum-infiltrated at − 80 Bar for 10 min, followed by incubation for an additional 10 min under atmospheric pressure to facilitate the penetration of bacteria into lenticels and wounds of the tubers. Inoculated potato tubers were air-dried overnight and, on the following day, vacuum-infiltrated with the mixture of five soft rot pathogens. Following the second round of vacuum infiltration, potato tubers were air-dried for 1.5 h and placed in covered, nontransparent plastic boxes, 30 tubers per box (box dimensions 26 × 18 × 12 cm). For the control treatments, tubers were inoculated with tap water and the path-ogens (positive control for the occurrence of soft rot, PC) or tap water alone (negative control, NC). Each preparation was tested in five technical replicates (n = 5 boxes × 30 tubers = 150 tubers). Boxes were stored for 6 months in a cold room, at 8 °C, 80% relative humidity, to simulate conditions applied for the storage of potato tubers (2–10 °C, depending on storage time and tuber type) (Beukema and van der Zaag 1990; Bradshaw and Ramsay 2009). Following the period of stor-age, the emerging sprouts, if present, were removed from the tubers, and the sprout-less tubers were incubated for 5 days under disease-favoring conditions (28 °C, 85–90% relative humidity), 15 tubers of the same treatment per box, to initiate soft rot. The temperature of 28 °C and 90% relative humidity were chosen to stimulate expression of soft rot symptoms caused by SRP on potato tubers. This experimental setup as-sured the worst case scenario, in which potato tubers of the relatively susceptible potato cultivar were challenged with high inoculum of the mixture of SRP pathogens under condi-tions stimulating the development of infection symptoms. The formulations containing the GF consortium able to protect tubers under disease-favorable conditions should confer their efficacy also under conditions less suitable for disease devel-opment (e.g., commercial storage conditions).

Severity of soft rot symptoms was assessed individually for each tuber using a six-rank disease severity scale (0–5) as previously described (Krzyzanowska et al. 2019a, b: 0—no symptoms, 1—rotting symptoms localized in the periderm and overall on less than 25% of tuber surface, 2—symptoms as in rank 1 but present on 25 to 50% of tuber surface, 3—symptoms as in rank 2 but with the periderm detaching from the internal tissue of the tuber (core) and the rotting occupying between 50 and 90% of the tuber, 4—symptoms as in rank 3 but overall the rotting occupies more than 90% of the surface and spreads to the core, and 5—maceration of the whole tuber. The experiment was performed twice. In total, each treatment was evaluated on 300 seed tubers, yielding a total of 1800 tubers for 6 treatments tested.

Statistical analyses

Statistical analyses were performed with R version 3.5.1 (RTeam 2018) using the RStudio (2017). For the evaluation the survival of cells following freeze drying, as well as for the shelf life experiment, the normality of distribution of the residuals was tested with Shapiro-Wilk test, and the homogeneity of variance was tested with Levene's test included in the "car" package (Fox and Weisberg 2010). For data with normal distribution and non-homogenic variance, as observed in case of the shelf life experiment, the differences between multiple groups were analyzed with Welch's unequal variances *t* test, followed by pairwise comparisons using Games-Howell test from "PCMRplus" package (Bürkner 2017). For data with non-normal distribution, as obtained in the experiment concerning the survival of cells following freeze drying, Kruskal-Wallis test (Kruskal and Wallis 1952) was used to determine the differences between samples, followed by a post hoc Dunn's test from the "dunn.test" package (Dinno 2017). The latter approach was also applied for the analysis of data in ordinal scale, like the ranks in disease severity scale (0–5) obtained in the biocontrol experiment on potato tubers. For data on the incidence of soft rot, expressed in binominal scale (0—lack of symptoms, 1—occurrence of symptoms), the Chi^2 test was applied to determine the difference between the expected and the observed frequencies between sample sets. For data from shelf life experiment, regarding survival at different temperatures, the normality of distribution of the data was checked with Shapiro-Wilk test. Due to non-normal distribution of data, Wilcoxon signed rank test was used to investigate differences between groups (Wilcoxon 1947).

Results

The GF strains show high survival rate following freeze drying in Reagent PS—a BSA-free alternative to the Reagent 18

The count of viable cells before and after the freeze drying procedure and the ratio was calculated to assess how the biocontrol strains of the GF consortium (*S. plymuthica* strain A294, *E. amnigenus* strain A167, *R. aquatilis* strain H145, *S. rubidaea* strain H440, and *S. rubidaea* strain H469) survive the freeze drying procedure and if they can be successfully preserved for long-term storage using this method. To provide a reference point, the ten other microorganisms were tested in parallel (4 probiotic bacteria, 1 probiotic yeast, 3 plant-associated bacteria, and the model *B. subtilis* strain 168 and *E. coli* strain DH5α).

The count of viable cells in the starting suspensions (prior to freeze drying) was approx. 10–11 $\log_{10}$ cfu g^{-1} (10^{10} to 10^{11} cfu $g^{-}1$) of cell fresh weight (fw) for all 14 bacterial strains and approx. 9 $\log_{10}$ cfu g^{-1} fw (1×10^9 cfu g^{-1}) for the single yeast strain tested. Following lyophilization, the positive effect of applying lyoprotectants was visible for 13 out of 15 tested microorganisms, providing cell survival between 6 and ca. 100 times higher in comparison to the survival of cells freeze-dried in water (negative control) (Supplementary Table S1). For the five biocontrol strains of the GF, the survival rate in Reagent 18 was ≥ 58% (average 73%) and in Reagent PS ≥ 57% (average 78%), and only 2–10% (average 7%) in water (Fig. 1), equaling a 1–1.7 drop in $\log_{10}$ cfu g^{-1} fw (Supplementary Table S2). The highest survival rate among the GF strains was obtained for *S. plymuthica* A294 and *S. rubidea* H440 in Reagent PS and for *S. rubidea* H469 in Reagent 18. In these cases, no loss of viable bacterial cells was detected (Fig. 1, Supplementary Table S1). At the same time, even with the addition of lyoprotectants, the survival rate of *P. protegens* CHA0, widely studied for its biocontrol properties, never exceeded 60%.

The most dramatic increase of survival upon the application of lyoprotectants was observed for the 3 tested probiotic *Lactobacilli*: two *L. rhamnosus* strains (GG and 573) and *L. brevis* 269Y. In case of these strains, the survival rate was improved 14–60-fold from 1 to 5% in water to 60–72% in Reagent 18; in comparison, almost no loss of viability was observed in Reagent PS. On the contrary, for *O. quorumnocens* A44, the increase in viability was less pronounced due to the high survival rate of the strain in water (37%). None of the tested conditions were suitable to efficiently preserve the yeast *S. boulardi* (ENTEROL 250 (BIOCODEX, Warsaw, Poland)).

Along with the comparison of the survival rate of individual strains, we evaluated the overall performance of two lyoprotectants: Reagent 18 and Reagent PS developed specifically for this study. The results showed that the protective effect of Reagent PS was not significantly different ($\alpha = 0.05$) from that of Reagent 18, with the average survival rates calculated for all 15 strains being 68% and 60%, respectively (Fig. 2). The average cell survival rate in water (11%) was significantly lower than that in Reagent 18 and Reagent PS ($p = 0.0001$ in both cases).

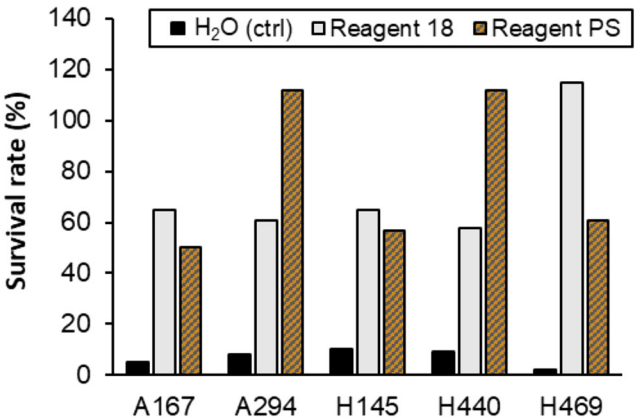

Fig. 1 Average survival rate of the tested GF strains following freeze drying in water (control) and in two lyoprotectants: Reagent 18 and Reagent PS. CTRL, control

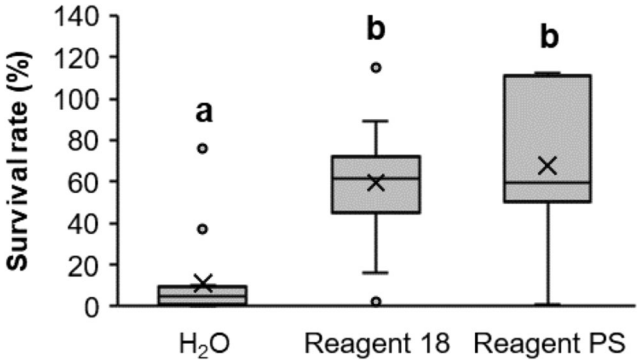

Fig. 2 Cell survival rate following freeze drying in water (control) and in the presence of two different lyoprotectants, Reagent 18 and Reagent PS. Data for 15 microorganisms were analyzed collectively. Significantly different groups (Dunn's test, $\alpha = 0.05$) are marked with different letters

The shelf life of the GF strains is higher in dry formulations stored at 8 °C than in liquid formulations stored at 22 °C

Long-term viability of the five GF strains, in different formulations, was evaluated for individual strains as well as for the five-strain consortium over a period of 12 months. The tested formulations included two liquid formulations: LQ (cells in ¼ Ringer's supplemented with the formulation mix) and CTRL (control for LQ; cells in ¼ Ringer's alone), and threepowders: LYO (lyophilizate), WP-KAO, and WP-DE. In total, 30 combinations were tested (5 individual strains plus the GF consortium (=6) × 5 formulations). Two independent experiments were carried out involving two different formulation lots (lot 1 and lot 2). The initial count of viable cells was between 9.6 and 10.5 $\log_{10}$ cfu mL^{-1} for the liquid formulations and between 10.0 and 11.9 $\log_{10}$-cfu g_{-1} for the powder ones (Supplementary Table S3). When evaluating cell survival, the absolute change in the number of cells between the starting value and the final value, as well as the linear regression slope (trend) values of the survival curves, were considered. The slope values express the average decline in cell count ($\log_{10}$-cfu g_{-1} or cfu mL^{-1}) per each month of storage. The lower the calculated slope value, the steeper decrease in the number of viable cells was observed. The comparison of the slope values, further transformed to lethality rate constants (k), is a good way to reliably compare the survival between different strains, formulations, and in different lots, irrespective of the initial variance in the cell count (Golowczyc et al. 2011).

Bacterial viability over time was compared at two temperatures: 8 and 22 °C, mimicking cold conditions predominantly used for potato tuber storage and room temperature, respectively. Gathering of data and evaluation of the results were performed at the fifth sampling time point, that is following 6 months of storage. At 8 °C, 17 out of 30 tested combinations (57%) showed less than one order of magnitude (1 $\log_{10}$ cfu g^{-1} or cfu mL^{-1}) drop in the count of viable cells, indicating a high survival rate in refrigerated conditions. At the same time point, the survival rate for all strains at 22 °C was statistically lower, with an average decline equaling nearly three orders of magnitude (2.7 $\log_{10}$ cfu g^{-1} or cfu mL^{-1}). At room temperature, a decrease ≥ 1 $\log_{10}$ cfu was observed for all 30 combinations (100%), and a decrease ≥ 2 log10 cfu was observed for as many as 22 (73%) (Supplementary Table S4). In line with the above data, the slope values for the survival of strains kept at 8 °C (− 0.17; average for all strains) was significantly higher ($p < 0.000001$) than those kept at 22 °C (− 0.41) (Fig. 3a), indicating a steeper decrease in the number of viable cells at 22 °C. Considering the effect of different temperatures after 6 months of storage, the monitoring of shelf life of the tested formulations at 22 °C was discontinued due to low cell survival. The monitoring of the viability at 8 °C was continued for up to 12 months.

Apart from analyzing the effect of temperature, we analyzed the influence of different formulation methods on the stability of formulated consortia. The ability of different formulations to assure high survival rate of the cells stored at 8 °C was compared using data pooled for all strains formulated in a given manner. In general, the survival rate of the GF strains in dry formulations (LYO, WP-KAO, WP-DE) was very good (Fig. 4) and, based on the comparison of the slope values, significantly higher than in the liquid preparations (LQ, CTRL) (Fig. 3b). From the tested formulation methods, the CTRL (bacterial suspension in ¼ Ringer's buffer alone) offered the lowest survival rates (− 0.28) and the lyophilizates (LYO) the highest (− 0.08) (Fig. 3b). The differences observed between the three dry formulations, LYO, WP-KAO, and WP-DE, were not statistically significant ($\alpha = 0.05$), and the two latter (WP-KAO and WP-DE) formulations offered a considerable reduction of dusting during development of the formulations and the subse-quent handling.

A strain-by-strain comparison of different formulations stored at 8 °C revealed that the most severe decline in the number of viable cells was observed for *S. rubidaea* H440 stored in ¼ Ringer's buffer (CTRL), with the slope equaling − 0.424 and a drop of 2–3.5 $\log_{10}$ cfu mL^{-1}. On the contrary, the combination offering the highest surviv-al rate was the same strain yet preserved in a dry form of lyophilizate (LYO) (average slope = − 0.037; drop ≤ 0.4 $\log_{10}$ cfu g^{-1}t following 6 months) (Supplementary Table S3).

To conclude, the most promising shelf life results were obtained for formulations containing desiccated cells stored under refrigerated conditions (8 °C).

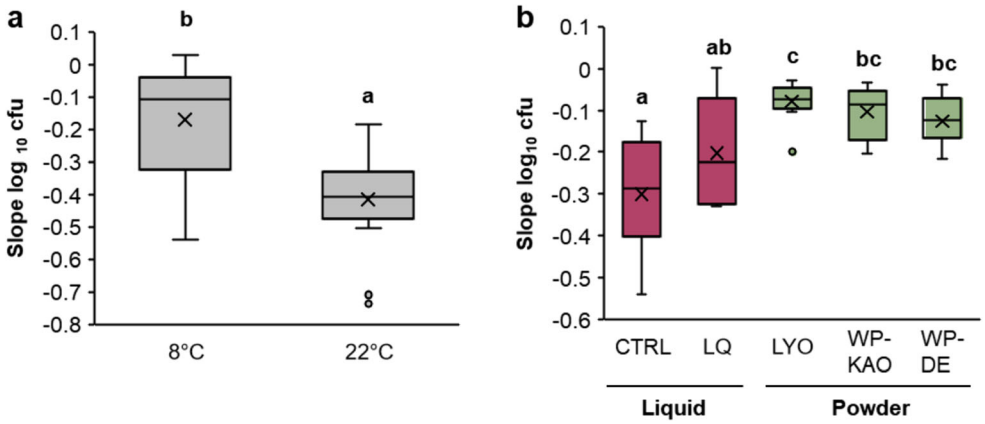

Fig. 3 The influence of temperature (**a**) and the formulation method (**b**) on the shelf life of the GF strains. The analyses were performed on data pooled for all five GF strains. Each box shows the slope of the survival curves (change in $\log_{10}$ cfu), calculated based on the count of viable cells (cfu mL^{-1} for the liquid formulations and cfu g^{-1} for the powders) for 5 time points in panel **a** (0, 1, 2, 3, and 6 months) and 7 time points in panel **b** (additionally 9 and 12 months, with the exception of LQ and CTRL from lot 1). The higher (closer to zero) are the calculated slope values, the better is the survival rate of the strains. Liquid formulations (red boxes): CTRL, positive control—cells suspended in ¼ Ringer's buffer (control); LQ—cells suspended in ¼ Ringer's supplemented with the formulation mix. Powder formulations (green boxes): LYO, bacterial lyophilizates; WP-KAO—bacterial lyophilizates with WPs formulation mix, kaolinite carrier; WP-DE—bacterial lyophilizates with WPs formulation mix, dia-tomaceous earth. Different letters indicate significant differences between groups (*t* test, $\alpha = 0.05$)

Powder formulations of the GF consortium significantly reduce the incidence and the severity of soft rot following 6 months of storage of inoculated potato tubers at 8 °C

To test if the formulated consortium of the GF antagonists can protect potato tubers in a setup mimicking the commercial storage conditions, tubers inoculated with both the GF and the combination of soft rot pathogens were stored for 6 months at 8 °C and subsequently transferred to disease-favorable con-ditions to initiate tuber rotting. A total of 2.7% of all tubers processed in Experiments 1 and 2 already showed symptoms already after their recovery from the cold room. These tubers were not transferred to disease-favorable conditions, which was the subsequent step of the experimental procedure. All tubers showing decay at 8 °C were considered as symptomatic and were assigned to the maximum severity rank 5.

Co-inoculation of potato tubers with the freshly grown GF antagonists (FR) reduced the average soft rot severity, in comparison to pathogen-only control (PC), by 94% in Experiment 1 and by 83% in Experiment 2. The protective efficacy of LYO, WP-DE, or WP-KAO was comparable between the three treatments and, although not as impressive as that of FR, still high, showing a 62–69% decrease in the average severity score in Experiment and 64–75% in Experiment 2 (Fig. 5a, b).

During the evaluation of disease incidence, only the tubers showing absolutely no disease symptoms were considered as healthy, whereas all other tubers were treated as symptomatic. In the pathogen-only control (PC), the soft rot incidence equaled 77% in Experiment 1 and 74% in Experiment 2. In comparison to PC, the application of FR reduced the disease incidence by 96% in Experiment 1 and by 82% in Experiment 2. Treatment with lyophilizate (LYO) reduced the incidence by 47% and 60%, and the two tested formulations, WP-KAO and WP-DE, resulted in a reduction of 48% and 59%, and 57% and 61% in comparison with the control, respectively (Fig. 5c, d.

Discussion

Although a number of attempts have been made to control SRP on potato using biological control agents, so far, none of them have resulted in development and commercialization of a microbial-based biocontrol product (Charkowski 2018; Czajkowski et al. 2011). Likewise, there are only few examples of the use of such bioproducts being successful in other bacterial pathogen-crop systems (Azizbekyan 2019; Nega 2014; Sheppard et al. 2003) The main reason for this is that for commercialization, the microorganisms need to be effectively formulated in order to remain viable and retain their properties throughout the storage period, in transport and upon application under environmental settings (Berninger et al. 2018).

This study was conducted to develop formulations provid-ing good shelf life of an artificial bacterial consortium termed "The Great Five" (GF) and to test the obtained formulated bacteria for protection of potato tubers against soft rot caused by SRP following long-term (6 months) storage at 8 °C, there-fore, mimicking storage conditions present in the commercial potato storage facilities. The biocontrol efficacy of the GF consortium was already reported in our former study, howev-er, only for freshly grown cells and only under short-term storage under disease-favoring conditions (Krzyzanowska et al. 2019a, b. The development of formulations and biocon-trol efficacy assays described herein was an important step on

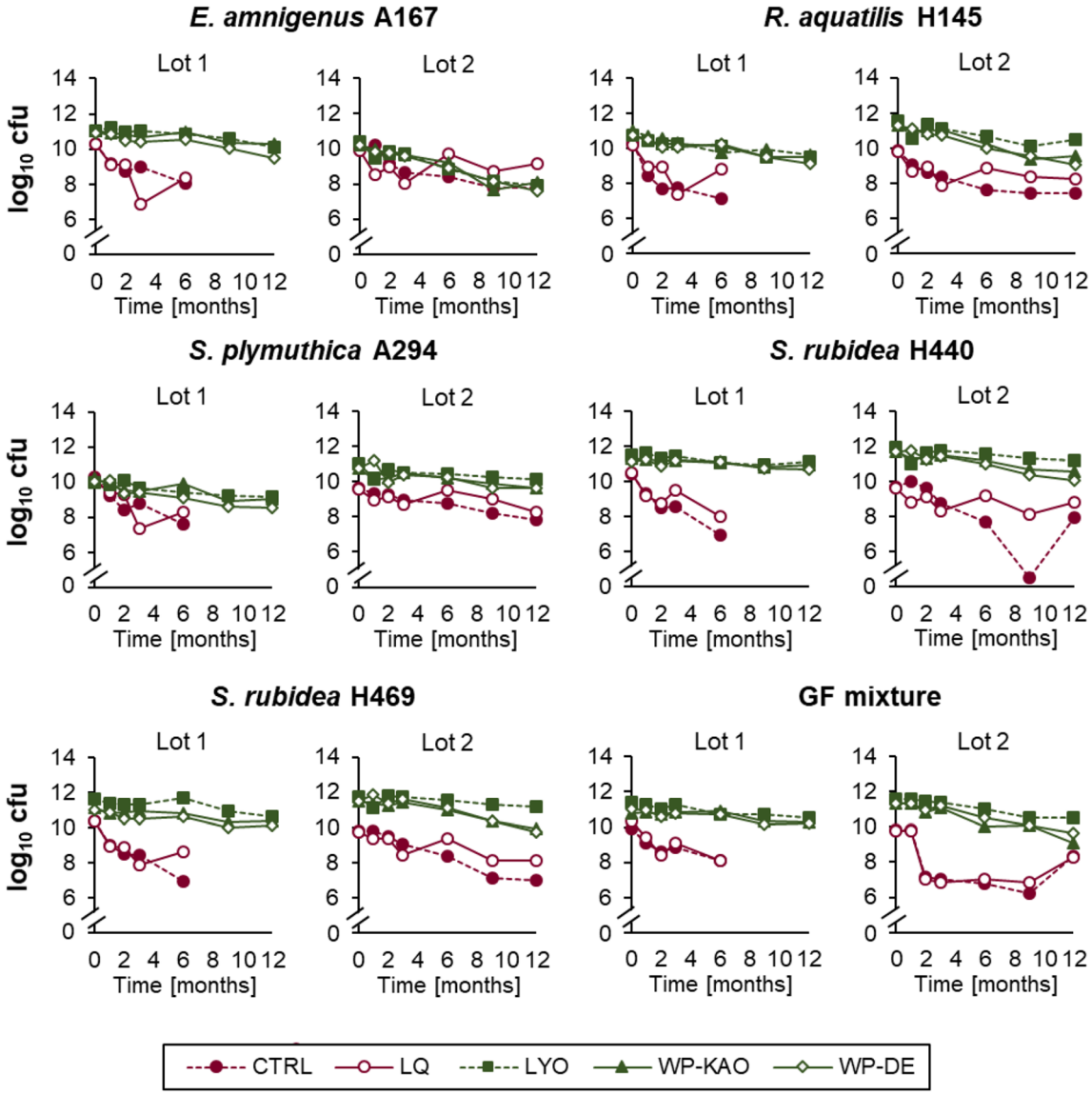

Fig. 4 The count of viable cells of the GF antagonists in different formulations stored at 8 °C for a total period of 12 months. Lot 1 and lot 2 refer to independent experiments in which different batches of formulations were tested. CTRL (control)—cells suspended in ¼ Ringer's buffer; LQ—cells suspended in ¼ Ringer's supplemented with LQ formulation mix; LYO—bacterial lyophilizates; WP-KAO—bacterial lyophilizates with WPs formulation mix, kaolinite carrier; WP-DE—bacterial lyophilizates with WPs formulation mix, diatomaceous earth. The sudden drop of the *S. rubidea* H440 $\log_{10}$ cfu in lot 2 observed between 8 and 10 months in control (CTRL) is an outlier happened due to the technical error. Another cell count, proceeding the 12-month time point (not shown in the Figure) in this treatment is in line with the assess-ment at the 12 month

the way to prepare "The Great Five" as a commercial product for agricultural applications, as suggested by others (Köhl et al. 2011).

To be eligible for practical application, formulations need to be prepared in a way which allows them to be handled via the standard distribution channels and/or under standard storage conditions (Leggett et al. 2011). This most often involves drying of the product and its storage in low humidity (Rhodes 1993). Desiccation of microorganisms can be carried out in several ways, e. g., freeze-drying, vacuum-drying, spray-drying, fluidized bed-drying, or air-drying (Broeckx et al. 2016); however, freeze-drying (lyophilization) is considered a meth-od of choice as it offers good survival rate, it is applicable both on large and small scale, and results in viable cells that can be rehydrated directly prior to use (Powell 1992; Berninger et al. 2018). The majority of microorganisms when freeze-dried without supplementation of lyoprotectants survive the process poorly (Heckly 1961).Viability rates as low as 0.1% have been frequently reported (Miyamoto-Shinohara et al. 2006).

One of the commonly used lyoprotectants, and the one recommended by American Type Culture Collection (ATCC, Virginia, USA), is Reagent 18. However, the use of

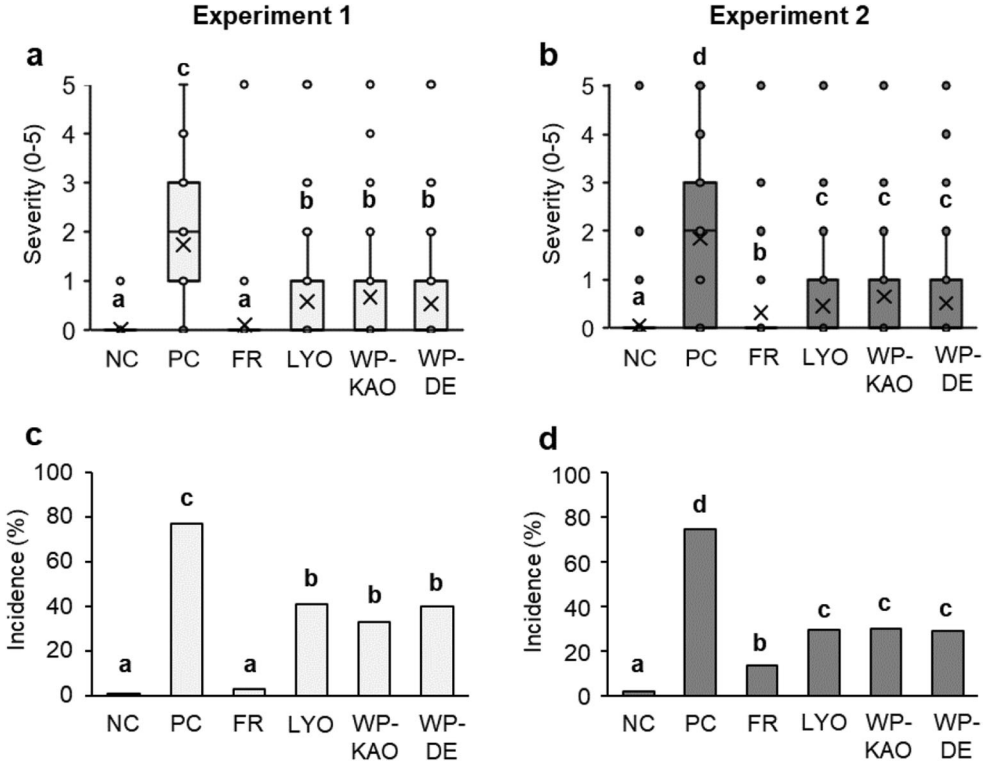

Fig. 5 Soft rot incidence (**a**, **b**) and soft rot severity (**c**, **d**) on potato tubers infiltrated with the GF antagonists and the SRP pathogens, followed by a 6-month storage at 8 °C. Results from two independent experiments, Experiment 1 (light gray) and Experiment 2 (dark gray) are shown sep-arately. The severity of symptoms was evaluated in a six-rank scale (0–5). NC negative control, tubers inoculated with water; PC positive control for the emergence of soft rot, tubers inoculated with SRPs alone. All other samples were co-inoculated with the GF antagonists and the SRP. Depending on the treatment, the GFs were delivered as: FR fresh cultures; LYO, lyophilizates; WP-KAO, lyophilizates formulated into a wettable powder with a kaolinite carrier; WP-DE, lyophilizates formulated into a wettable powder with a diatomaceous earth carrier. In the box plots **c** and **d**, each box determines the inter-quartile range (Q1–Q3), the line indicates the median value, the "×" stands for the average value, the whiskers indicate extreme values within 1.5 times distance from Q1–Q3, and single data points are outliners. In each experiment, a given combination was tested on 150 tubers. Different letters indicate significant differences (α = 0.05) between groups in Dunn's test (a, b) or Chi^2 (c, d)

Reagent 18 has limitations resulting from the fact that it contains bovine serum albumin (BSA). BSA is an expensive additive, significantly increasing the total cost of Reagent 18. Moreover, it is of animal origin which may lead to ethical concerns. In this study, to overcome these limitations, we developed Reagent PS as a more economically sound and ecofriendly alternative for Reagent 18. In an evaluation exper-iment performed on 15 microbial strains, the overall survival rate of cells freeze-dried in Reagent PS reached ca. 40–60%and was comparable to that obtained for Reagent 18 (Fig. 1, Supplementary Table S1), while only ca. 5–10% of microor-ganisms survived without a lyoprotectant (control). Survival rate of ca. 50% was previously reported as a measure of a successful lyophilization (Bozoğlu et al. 1987).

In the course of this study, the biological control strains of the GF consortium were lyophilized in the newly designed lyoprotectant Reagent PS and subsequently formulated, both as individual strains and as the GF consortium, into two pow-der formulations. Powder formulations have the widest appli-cations in bioproducts as they can be applied directly on the plant material or, in case of wettable powders, suspended in water and applied as a water-based suspension (Boyetchko et al. 1999). For safety reasons, we selected the latter method of formulation and application. This method is also preferred by farmers as dusting may be hazardous for the workers (Knowles 2008). Although the additives applied to the formulated cells in this study did not offer prolonged shelf life of microorganisms compared with the lyophilizates alone, they considerably reduced their electrostatic properties and dusting, therefore increasing the ease and safety of handling.

As reported earlier, powder formulations in general offer better shelf life than liquid preparations—bacterial cells remain viable for longer periods and the survival is higher than in the other forms of formulations. A crucial factor for successful storage is also the storage temperature. Here, we observed a drastic decline (at average 2.7 $\log_{10}$ cfu in the first 6 months) in the viability of the studied strains when the formulations were stored at 22 °C. Storing the product at room temperature is an attractive, cost-efficient option, however rarely possible in case of biological plant protection products. Similar decline of viability in formulated bacterial cells at 22 °C was reported for other biological control agents,

including *Pseudomonas fluorescens* EPS62e and *B. subtilis* CPA-8 (Cabrefiga et al. 2014; Yánez-Mendizábal et al. 2012).

In contrast, the GF strains formulated as wettable powders and stored at 8 °C survived for a period of 12 months without a significant drop in cell numbers. Most of the bioformulations currently available on the market have a declared shelf life of 1 year, with minimum of 3 months and up to 6 years in case of selected spore-based products (Arora and Mishra 2016; Preininger et al. 2018). Suggested storage conditions include freezing (− 20 °C), cooling (4–10 °C), or room temperature depending on the type of formulation and its content. This implies that the strains of the GF consortium, formulated to wettable powders as described herein, present shelf life ac-ceptable for commercial products.

Since storage conditions can either positively or negatively influence the activity of biocontrol agents, it is of utmost importance to test bacterial formulations under the appropriate conditions mimicking the real life situation (Costa et al. 2002; Qin et al. 2004). The formulations of GF strains were therefore tested for their biocontrol efficacy against SRP on potato tubers stored for 6 months at 8 °C and under 80% relative humidity. These experimental settings simulate standard (commercial) conditions of potato tuber storage (Bradshaw and Ramsay 2009). For this work, tubers were subsequently inoculated with antagonists and SRPs, stored at 8 °C for 6 months, and then transferred from storage to disease-favorable conditions (28 °C and 85–90% relative humidity) to initiate soft rot symptoms. In this setup, formulations containing antagonistic bacteria decreased symptoms caused by SRP by 50%. Freshly grown cells of the GF consortium, used as reference, offered higher efficiency of protection than formulated bacterial cells of comparable inoculum size. This, however, was not unexpected. It is possible that after rehydration, some strains in the formulations failed to multiply because of their physiological condition. In our study, formulations comprising the GF consortium were freshly prepared from lyophilizates of single bacterial strains. The viable cell count in lyophilizates was determined by dilution plating. During inoculation of potato tubers, water-rehydrated bacterial cells went directly to a poor environment (potato surface, potato skin, and lenticels). We presume that freshly grown cells, coming from optimal growth conditions in a rich medium, may behave differently in this situation than the previously dormant, formulated cells. Similar observations have been made in cases of other formulated biological control agents (Berninger et al. 2018).

Literature suggests that application of microbial consortia, either composed in the laboratory or selected as functional units directly from the environment, may provide a good strategy to develop efficient biocontrol agents (Droby et al. 2016; Fukui et al. 1999; Meyer and Roberts 2002). Currently, the major factor limiting smooth introduction of such products on the market are regulations concerning registration of biological plant protection products, especially in the European Union (Frederiks and Wesseler 2019). According to these regulations, in case of multi-strain products, each active component (strain) should be evaluated separately, sig-nificantly increasing the cost, time, and effort necessary to go through the registration procedure, in principal, designed for chemical agents. To register a (bio)product, the applying en-tity needs to provide data on potential toxicity and ecotoxicity of the product. Currently, there is a strong lobby to alleviate the requirements for registration of biopesticides which, alike bacteria present in the GF consortium, are the elements of the natural microbiome of the soil and/or various plants and which are, according to the current knowledge, not harmful to humans. In the light of growing demand for ecological prod-ucts for sustainable agriculture (Arora et al. 2016), as well as scientific data on the benefits of applying microbial consortia, it is therefore important that the regulations be adapted to this new generation of products and microbiological agents in general.

In conclusion, in this, study we provided evidence that the newly developed formulations of "The Great Five" micro-consortium, when stored at 8 °C, assure good shelf life of at least 1 year. Moreover, the preserved cells retain their antago-nistic activity towards SRP on potato tubers. Further studies are required to optimize the process of application of the for-mulations under storage conditions. Other matters worth ad-dressing also include the potential of the GF consortium to control SRP under field conditions as well as the longevity of the applied micro-consortium in soil to assure protection on growing plants. Finally, elucidation of the molecular mecha-nism of antagonism could be of value for using the micro-consortium in other pathogen-host combinations.

Authors' contribution statement

TM, DMK, JS, and RC: investigation and methodology; TM, DMK, SJ, and RC: writing, reviewing, and editing the manu-script; TM and DMK: data curation and project administra-tion; SJ and RC: resources; TM, DMK, and JS: visualization; RC: project administration. All authors approved the final version of the manuscript.

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Compliance with ethical standards

Ethical statement This article does not contain any studies with human or animal participants performed by any of the authors.

Conflict of interest The authors declare that they have no competing interests.

Disclaimer Reagent PS described herein, for protection of microbial cells during lyophilization, is the object of the patent application P.428215, which has been filed with the Polish Patent Office by University of Gdansk, Poland, with inventors Robert Czajkowski, Dorota Krzyzanowska, Tomasz Maciag, Joanna Siwinska, and Sylwia Jafra.

The method of formulating bacterial strains into the bioproduct and the application of the developed bioproduct in agriculture described herein is the object of the patent application P.431434 which has been filed with the Polish Patent Office by University of Gdansk, Poland with inventors Robert Czajkowski, Dorota Krzyzanowska, Tomasz Maciag, and Sylwia Jafra.

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2.3. Complete Genome Sequences of Five Gram-Negative Bacterial Strains Comprising Synthetic Bacterial Consortium "The Great Five" with Antagonistic Activity Against Plant-Pathogenic Pectobacterium spp. and Dickeya spp.[1]

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Short description

This manuscript describes the genomes of the strains comprising the artificial consortium active against Soft Rot Disease. It is vital to analyze the genomes of bacteria which are to be used in agriculture to indemnify potential threads to human health (Deising et al., 2017). Additionally, analyzing the genes of active biocontrol agents can help identify the most important features for such strains and help find new candidates (Loper et al., 2012). Although microbial consortia potentially have more stable performance, the strains mixed together can sometimes diminish activity (Stockwell et al., 2011). Therefore, it is important to identify features important for strain compatibility to help design new compatible consortia (Johns et al., 2016).

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RESOURCE ANNOUNCEMENT

Complete Genome Sequences of Five Gram-Negative Bacterial Strains Comprising Synthetic Bacterial Consortium "The Great Five" with Antagonistic Activity Against Plant-Pathogenic *Pectobacterium* spp. and *Dickeya* spp.

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Genome Announcement

There is a growing interest in using synthetic microbial consortia as biological control agents (biopesticides) in agricultural applications (Arora et al. 2016; Mehnaz 2016). This interest is manifested because microbial consortia can offer higher reproducibility of biological control under various environmental conditions and provide a broader array of modes of action than any individual biological control agent applied alone against the given pathogens (Mishra and Arora 2016).

However, despite the global demand for better-performing, more environmentally friendly crop protection systems in agriculture, there still are very few biopesticides on the market comprising more than one active microbial biological control agent. The reasons for that situation are diverse (Vishwakarma et al. 2020); however, two critical challenges may be identified. First, there are unresolved issues with the registration and marketing of such bioproducts, which limit their potential use in modern agriculture on a large scale. Second, the difficulties in understanding the specific roles of each component of a microbial consortium and their biological activity may limit the predicted final protective effect on the crop (Czajkowski et al. 2020). Due to the above, more research is required concerning microbial consortia with biological control activity and their feasibility in agricultural applications (Xu et al. 2011). To address this issue, we sequenced the genomes of bacterial strains comprising the "Great Five" (GF) synthetic microbial consortium effective against potato soft rot disease caused by *Pectobacterium* and *Dickeya* spp. (Krzyzanowska et al. 2019; Maciag et al. 2020).

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e-Xtra: Supplementary materials are available online.

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Keywords

agriculture, biological control, blackleg, genomics, plant pathogen, soft rot, syn-com, synthetic consortia

This GF synthetic bacterial consortium comprises five strains: *Lellilottia amnigena* (former *Enterobacter amnigenus*) strain A167, *Serratia plymuthica* strain A294, *Rahnella aquatilis* strain H145, *S. rubidaea* strain H440, and *S. rubidaea* strain H469 (Krzyzanowska et al. 2019). Strains of *L. amnigena* A167 and *S. plymuthica* A294 were isolated from the potato rhizosphere (Jafra et al. 2006). In contrast, strains of *R. aquatilis* H145 and *S. rubidaea* strains H440 and H469 were isolated from the inside of hyacinth bulbs (Jafra et al. 2009). When combined into the GF consortium, the five bacterial strains were shown to suppress soft rot symptoms on potato tubers in storage, even under high pathogen pressure and conditions favoring disease progression (Krzyzanowska et al. 2019). Furthermore, the GF consortium has been successfully formulated into a stable product that can be readily applied in potato production systems (Maciag et al. 2020). The latter is essential in transferring to an agricultural setting (Bashan et al. 2014).

The complete genome sequences of the strains comprising the GF synthetic consortium can help to identify features essential for the biocontrol activity of the consortium, interactions between the strains, and strain compatibility, as well as the safety of use in agricultural applications (Deising et al. 2017; Loper et al. 2012; Stockwell et al. 2011).

For genomic studies, bacterial DNA was isolated using the Wizard Genomic DNA purification KIT (Promega Corp.) and additionally purified with the Clean NA kit (GC Biotech b.v.) according to the instructions provided by the manufacturers. Genome sequencing was performed in parallel by two platforms: Illumina Mini-Seq and Oxford Nanopore Technology. The raw reads of each strain of the consortium produced during genome sequencings were deposited in the NCBI Sequence Read Archive under BioProject PRJNA557569 and accession number SRP363301. Data were de novo assembled using Unicycler v0.4.8, with a final mean coverages of 287.2$\times$ for A167, 199.8$\times$ for H145, 271.8$\times$ for A294, 258.9$\times$ for H440, and 259.8$\times$ for H469. Initial genome polishing was conducted by a tool integrated into the assembler (Racoon polishing script). Pilon 1.23 was used to further correct the errors. After that, Illumina reads were mapped to contigs from previous steps. Visual inspection was done on mapped files using Geneious Prime 2020. The procedure was done to check for any drop in coverage that could happen in repeated regions (manual validation) and to close any open contig that was not correctly assembled due to the multiplication of the same sequence on both contig ends (manual curation).

Each time, the combined procedure produced a single contig (Table 1). The GF genomes were annotated with the NCBI Procaryotic Genome Annotation Pipeline (Tatusova et al. 2016). The obtained genome sequences of 4.5 to 5.5 Mbp in length were deposited in the NCBI GenBank database. The GenBank accession numbers, genome sizes, and GC contents of each GF strain are given in Table 1. Interestingly, we found that *R. aquatilis* H145 possesses three plasmids: two of 500 kbp and one of 115 kbp. *L. amnigena* A167 has one 109-kbp plasmid, and each *S. rubidaea* strain has one 3.5-kbp plasmid (Table 1). The four genomes H145, A167, H440, and H469, contained plasmids in addition to the main replicon.

The sequenced genomes were compared based on the composition of the Cluster of Orthologous Groups assigned by eggNOG (Huerta-Cepas et al. 2017). All strains had approximately 40% of genes involved in general (primary) metabolism and 20% in each of the following groups: cellular processes and signalling, information storage and processing, and poorly characterized. Comparing the percentage of genes from different orthologous groups, *L. aminigena* A167 differs the most from the other strains of the GF consortium. A167 has more genes responsible for inorganic ion transport and metabolism and fewer genes accountable for amino acid transport and metabolism (Supplementary Table S1).

The AntiSMASH 6.0.1 (Blin et al. 2021) platform was used to analyze genomes for the presence of genes involved in secondary metabolism (production of antibacterial or antifungal secondary metabolites). Analyses were performed with KnownClusterBlast, ClusterBlast, SubClasterBlast, ActiveSiteFinder, Cluster Pfam analysis, and Pfam-based gene ontology term annotation features (Table 1). For *S. plymuthica* A294, the algorithm detected 14 clusters involved in secondary metabolism, 6 belonging to nonribosomal peptide synthetases (NRPS), 1 poliketyde synthase (PKS), and 2 siderophore clusters. In addition, two of the found clusters have a 100% similarity with clusters responsible for the synthesis of sodorifen and zeamine. For *L. amnigena* A167, antiSMASH 6.0.1 detected

Table 1. General features of the genomes of five antagonistic strains belonging to the "The Great Five" consortium

Species, strain, source, accession	Size (bp)[a]	GC content (%)	CDS[b]	Clusters involved in the synthesis of secondary metabolites[d]				
				Total (metabolite)[c]	NRPS	PKS	Sid	Bact
Lellilottia amnigena, A167, potato rhizosphere								
CP042361	4,520,659	52.9	4,229	2 (arylopyene)	1	0	0	0
CP042362 (plasmid)	109,787	64.2	140	–	–	–	–	–
Rahnella aquatilis, H145, hyacinth bulb								
CP042357	5,033,524	51.7	4,633	5 (desferrioxamine E, xanthoferrin)	1	0	2	0
CP042358 (plasmid)	536,322	51.7	486	–	–	–	–	–
CP042359 (plasmid)	467,533	49.6	467	–	–	–	–	–
CP042360 (plasmid)	111,445	52.9	115	–	–	–	–	–
Serratia plymuthica, A294, potato rhizosphere								
CP042363	5,534,595	56.2	5,085	14 (sodorifen, zeamine)	6	1	2	0
S. rubidaea, H440, hyacinth bulb								
CP042355	4,952,316	59.2	4,596	12 (pyrrolnitrin)	4	1	4	2
CP042356 (plasmid)	3,461	45.0	5	–	–	–	–	–
S. rubidaea, H469, hyacinth bulb								
CP042353	4,952,534	59.2	4,595	12 (pyrrolnitrin)	4	1	4	2
CP042354 (plasmid)	3,461	45.0	5	–	–	–	–	–

[a] Genome size. Accession numbers of replicons are given according to size from chromosome to the smallest plasmids for each strain.
[b] Coding sequence (CDS) counts.
[c] Total (predicted secondary metabolite. Only clusters with score ≥50% (similarity in amino acid sequence to known clusters ≥50%) are listed in the table.
[d] Clusters responsible for secondary metabolism were assigned with antiSMASH 6.0.1. NRPS = nonribosomal peptide synthetase, PKS = poliketyde synthase, Sid = siderophore, and Bact = bacteriocins. Analyses were performed with KnownClusterBlast, ClusterBlast, SubClasterBlast, ActiveSiteFinder, Cluster Pfam analysis, and Pfam-based gene ontology term annotation features turned on. Some clusters can be assigned to more than one category; for example, NRPS siderophores.

only two clusters: one NRPS cluster and one region with 100% similarity to a cluster involved in the synthesis of arylopyene. For *R. aquatilis* H145, five clusters were detected: one NRPS cluster, two siderophore regions, one region with 100% similarity to the cluster involved in desferrioxamine E synthesis, and another with 57% similarity to a region for the synthesis of xanthoferrin. For *S. rubidaea* strains H440 and H469, 12 clusters were detected: 4 NRPS, 1 PKS, 4 siderophore regions, 2 bacteriocins, and 1 region with 100% similarity to a cluster involved in the synthesis of pyrrolnitrin.

We have not found clusters encoding any secondary metabolites toxic to humans and animals in the obtained genomes, suggesting that the respective GF strains do not produce such compounds. These results, however, should be experimentally confirmed.

In turn, we found secondary metabolites with known antifungal activity, such as pyrronitrin (Arima et al. 1964), and antibacterial activity, such as zeamine (Hellberg et al. 2015). Therefore, the obtained data can help identify a broader use for the tested GF synthetic consortium; for example, against important potato pathogens other than *Pectobacterium* and *Dickeya* spp., including fungal pathogens such as *Rhizoctonia solani* (Jung et al. 2018). In addition, sodorifen produced by *S. plymuthica* is considered essential for interspecies communication by volatile compounds (Domik et al. 2016). Volatile-based communication may lead to changes in the profile of produced secondary metabolites and, therefore, changes in the antimicrobial activity of the GF consortium (Kai and Piechulla 2018; Schmidt et al. 2017).

The complete genomes of the biological control strains provide a valuable reference for research on understanding the traits essential in the consortium's interspecies interactions and their further applications in agriculture. The genome sequences of strains forming the synthetic GF consortium will help in the development and use of similar consortia for agricultural applications, allow a better understanding of the consortium's interspecies interactions, and lead to new applications for the already designed consortium.

Author-Recommended Internet Resources

Racoon polishing script: https://www.biostars.org/p/463148/
Pilon 1.23: https://github.com/broadinstitute/pilon/releases/
Geneious Prime 2020: https://www.geneious.com

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3. Discussion

This series of three experimental manuscripts show the path from idea to product, and how this research compares with other scientific works. Here, since it is a series of publications, I will try to put into perspective the whole experimental path rather than each result which was discussed in the enclosed manuscripts. A doctoral thesis, especially in the form of a series of articles, allows a broader look at the subject instead of the views presented in the experimental articles. I hope this approach will be both exciting and enjoyable for the reader and provide an original view on the presented matters.

3.1. Summary

There are many approaches to designing microbial consortia for biological plant protection. Swenson et al. divide these approaches to "top-down" and "bottom up". where in "top-down" approach, the known microbial strains are combined to obtain the desired function and "in bottom-up" approach, the consortia possessing desired activity are isolated and tested (Swenson et al., 2000). Most microbial consortia tested for biological plant protection are selected to combine different functions, especially when there are only two components (Sarma et al., 2015). Incorporation of more components though promising, may lead to the problem of strain incompatibility (Batra et al., 2020). This results in the limited number of consortia comprise three and more components (Mishra & Arora, 2016).

To tackle this problem, the consortium candidates are tested *in vitro* for growth inhibition of other components of the consortium. Although this is a generally accepted approach, it may raise some specific issues (Thomloudi et al., 2019). Firstly, the antibiosis of certain strains will depend on the medium used for testing, e.g. carbon source (Matuszewska et al., 2021), and bacteria grown on rich media produce different spectrum of antimicrobials than in nature (Sanders et al., 2018). Additionally, the incompatibility may not lay in the antibiosis between strains but in the degradation of substances responsible for the activity of certain strains (Stockwell et al., 2011).

Therefore, one can implement a different approach to design consortia with more components. It has been discovered that upon stress, plants can actively recruit beneficial strains (Rudrappa et al., 2008). This phenomenon can be used to isolate microbes for specific purposes already in reduced consortia (Mueller & Sachs, 2015). Alternatively, multispecies natural consortia are isolated and cultured together (Mehnaz, 2016; Preininger et al., 2018). This approach, however, will make future registration of biocontrol products more difficult since, among other things, the requirement to reveal the mode of action of such products (Arora et al., 2016; Frederiks & Wesseler, 2019).

In order to be able to quickly identify commponents of consortiums and secure a patent for the usage of a given consortium, we decided to compose the artificial consortium. The tested strains were previously isolated for their potential for biocontrol (Jafra et al., 2006, 2009; Krzyzanowska et al., 2012), and tested in different combinations for the activity to ensure the compatibility between strains. This novel approach has the advantage of being able to fully sequence and describe the components of consortium (Maciag et al., 2022), while not requiring the genetic modifications to maintain compatibility (Stockwell et al., 2011).

Another important aspect of using bacteria as biocontrol agents is their proper formulation to ensure good shelflife and physical properties (Bashan et al., 2014). Depending on the type and application of the microorganism, different formulations can be used: simple solutions, emulsible concentrates, dust powders and wettable powders (Knowles, 2008). We have selected two wettable powders and one liquid formulation for the simplicity of obtaining these formulations and the safety of use (Maciag et al., 2020).

Finally in order to identify potential hazards of using scented microorganisms from e.g. production of toxins (Deising et al., 2017), the strains were sequenced and their genome annotated (Maciag et al., 2022). The genetic information can be further used for identification of the features important for biocontrol (Loper et al., 2012) and compatibility (Johns et al., 2016).

3.2. Biological Control Based on Microbial Consortia – From Theory to Commercial Products [1]

1 Reprinted from: Robert Czajkowski, Tomasz Maciag, Dorota M. Krzyzanowska & Sylwia Jafra, 2020, Biological Control Based on Microbial Consortia – From Theory to Commercial Products. In: Antonieta De Cal, Paloma Melgarejo, Naresh Magan (Editors) How Research Can Stimulate the Development of Commercial Biological Control Against Plant Diseases. Progress in Biological Control, vol 21. Springer, Cham. https://doi.org/10.1007/978-3-030-53238-3_12

Short description

This book chapter describes the usage of microbial consortia for biological plant control. Here, we presented the biological bases of the consortia activity, the scientific research on the usage of microbial consortia for plant protection, what products for biological plant protection containing microbial consortia are available on the market, and the future perspectives in the field. This chapter summarizes the rationale behind the doctoral project, describes the research in the field comparing presented experimental work with other approaches and finally outlines the future of the research in this area.

Chapter 12
Biological Control Based on Microbial Consortia – From Theory to Commercial Products

Robert Czajkowski, Tomasz Maciag, Dorota M. Krzyzanowska, and Sylwia Jafra

12.1 Introduction

Biological control based on individual microorganisms (monocultures, clonal populations) and/or their products to be used in agriculture and industry is receiving an increasing attention worldwide (Baker 1987; Lewis and Papavizas 1991; Ferron and Deguine 2005; van Lenteren et al. 2017). Contrary, for many years the use of combinations (mixtures, cocktails) comprising several biological control agents – so-called: (synthetic or artificial) microbial consortia of bacteria, fungi as well as bacteria together with fungi has been neglected, mainly due to the problems occurring during registration and marketing (Woo and Pepe 2018). Likewise, these synthetic microbial consortia were not routinely used due to the difficulties in understanding the specific roles of each component of a consortium as well as their biological activity (Mittal et al. 2017).

This scarce number of the applications utilizing synthetic microbial consortia in agriculture may be a bit of a surprise taking into account that natural microbial mixtures/communities have been used as co-cultures for thousands of years for the food and beverage preparations (Ray and Didier 2014) and more recently also for composting, bioremediation and treatment of wastewater (Brenner et al. 2008; Brune and Bayer 2012). Likewise, it has always been expected that combinations of biocontrol strains result in a higher level of potential to suppress multiple plant diseases than the use of individual agents (Baez-Rogelio et al. 2017).

Very recently several studies demonstrated the usefulness of synthetic microconsortia composed specifically to increase the biological control activity against

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pathogens in agriculture (Kong et al. 2018; Bradáčová et al. 2019; Krzyzanowska et al. 2019). In these studies the emphasis has been laid to combine several biocontrol agents possessing different mechanisms of antagonistic activity in order to assure consistent performance of control against multiple pathogens and under a range of environmental conditions (Johns et al. 2016). This new approach tended to emulate the structured microbial networks of native suppressive soils in which the beneficial microbial groups positively influence soil fertility and pathogen control in a large scale (Raaijmakers and Mazzola 2016). Similarly, these synthetic micro- consortia may be viewed as moderators of the natural soil microbiomes diminished by crop domestication and/or as compositions establishing long-lasting associations with the natural soil inhabitants increasing the total biological control capacity of the particular environment (Puentes-Téllez and Falcao Salles 2018; Zegeye et al2019).

Selection of the members of synthetic micro-consortia is not a trivial task as it requires identification, culturing, compatibility analyses, ecotoxicological and performance tests *in situ* both for each member individually and for group of strains to be used (Julien-Laferrière et al. 2016; Ben Said and Or 2017). Although, there are not universal protocols describing selection of the members of optimal (synthetic) mixture, there are some common rules that can be used to establish and effective and long-lasting artificial microbial consortium for agricultural applications. For example, the chosen microorganisms should be nonpathogenic to humans, animals and plants, remain resistant to the adverse environmental conditions, active synergistically and fast, should possess long shelf-life, be easy to handle as well as cheap to produce (Nemergut et al. 2013; Wright et al. 2019).

Despite the fact that a number of microbial species can be considered as members of synthetic micro-consortia, the vast majority of current applications employ, at the maximum, only two species (dual/binary cultures) and consortia containing more than two members are rarely used in commercial agricultural and industrial applications (Woo and Pepe 2018). The dual synthetic consortia are usually exposed to the same conditions (temperature, pH, nutrient availability) because their members grow together in one common medium. The obvious, biggest limitation of this approach is the need for environmental and production conditions compatible for each of the two members (Maiyappan et al. 2010). This problem has to be taken into consideration as may expand when additional members of the consortium are included in the mixture.

From the agricultural perspective, the introduction of artificial micro-consortia to the rhizosphere or bulk soil may result in activation of various processes governed by both plant and/or microorganisms present naturally in the same niches (Tengerdy and Szakács 1998). These processes may include nitrogen fixation, solubilization of phosphate, production of phytohormones, siderophores and exopolysaccharides, resistance against drought, frost and humidity as well as production of antimicrobial substances directly limiting pathogen survival and growth (Syed Ab Rahman et al. 2018).

This chapter aims to provide insight into current state of the art in the development of the artificial microbial consortia for the agricultural applications mainly for

plant protection against pathogens and for biofertilization to increase plant fitness and crop yield.

12.2 Unraveling the Mode of Action of Individual Biological Control Strains and Microbial Consortia

Selection of biological control agents is most often based on a mode of action of the selected isolates and/or on their overall efficacy in plant (agricultural) environment (Köhl et al. 2011). The mode of action may in turn depend on a direct effect on pathogens' growth (antibiosis resulted from production of antibiotics, toxins, lytic enzymes, micro-parasitism) (Woo and Lorito 2007; Raaijmakers and Mazzola 2012; Krzyzanowska et al. 2016) or on an indirect action (e.g. induction of host resistance, competition for niche or interference in the cell-to-cell communication) (Höfte and Bakker 2007; Czajkowski and Jafra 2009; Annapurna et al. 2013). Some literature data also includes plant growth promotion (e. g. production of growth regulators, solubilization of phosphates, nitrogen fixation) as an important feature of biological control agents (Fig. 12.1).

Other features important for effective biocontrol agents are connected with the ability to colonize plant tissues or to form biofilm in plant surroundings (e.g. on

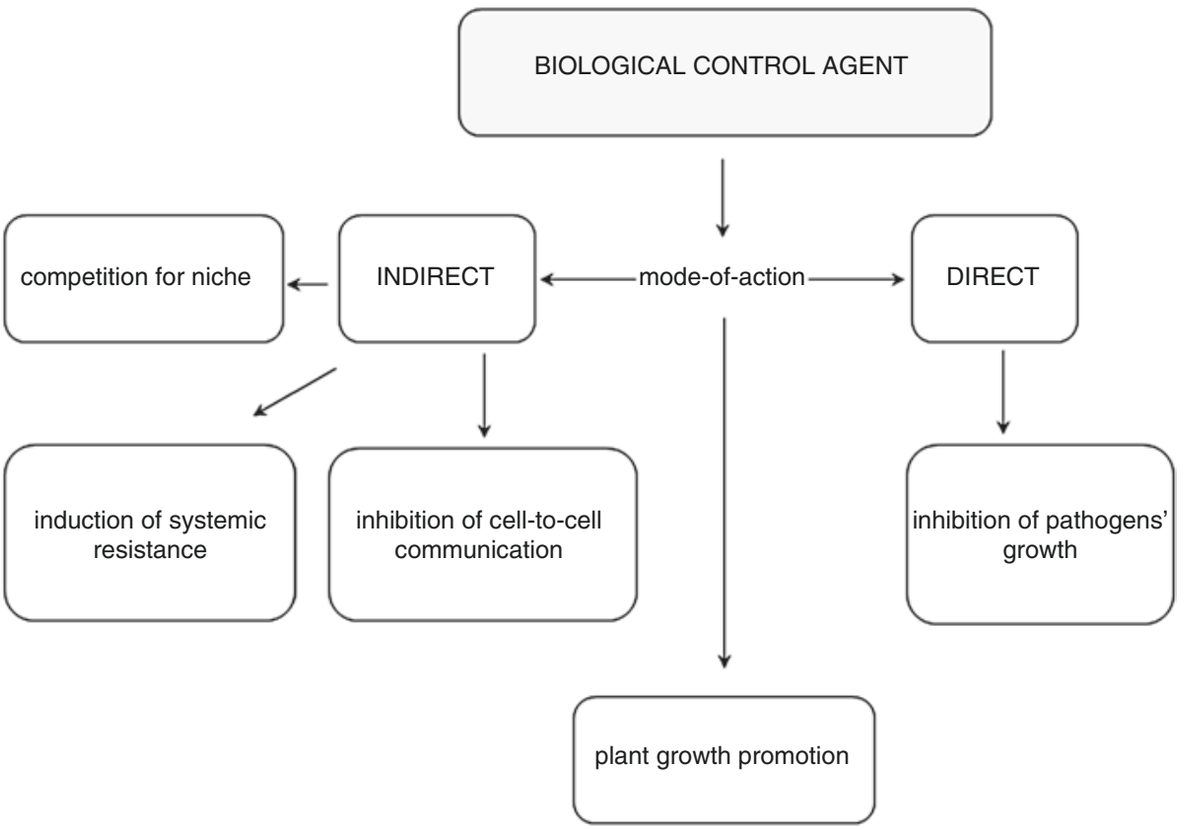

Fig. 12.1 The expected mode-of action of microorganisms recognized as biological control agents (BCAs)

roots, inside vascular tissue of stem, on leaves and other plant organs) (Thomashow and Bakker 2015), the production of volatile organic compounds with antimicrobial and signaling characteristics (Ossowicki et al. 2017; Tyc et al. 2017) and/or scav-enging iron ions from the plant environment (production of siderophores) (Kloepper et al. 1980; Höfte and Bakker 2007). Taking into account the area in which the BCAs should be active, the plant colonization or biofilm formation *in situ* is of the highest importance for biological control applications. It is known by now that the effective colonization of plant by beneficial endophytic microorganisms signifi-cantly increase plant fitness (Vinale et al. 2008; Pliego et al. 2011; Mercado-Blanco 2015; Afzal et al. 2019).

The successful plant colonizers benefit from plant protection while occupying the niche which gives relatively better access to nutrients than in the case of micro-organisms present in bulk soil.

A number of investigations aimed to identify microbial traits important and use-ful for biological control was done through reverse genetic approach; usually by selection of microbial mutants lacking the biocontrol phenotypes observed in the wild type and the analyses of the genetic background of the antagonistic activity (Silby and Levy 2004; Jackson et al. 2013; Vacheron et al. 2014; Krzyzanowska et al. 2016). The importance of motility, chemotaxis to root exudates, efficient nutri-ent uptake, vitamin and amino acids synthesis or utilization of organic acids are most often stated as essential for effective root colonization. The same attributes remains important also for biofilm formation as evidenced by other authors (Souto et al. 2004; Martínez-Gil et al. 2013).

Most often under laboratory conditions the individual isolates are analyzed for each possible mechanism (mode of action) separately (e.g. pathogen growth inhibi-tion in *in vitro* assay) – the approach that is difficult to apply for microbial consortia under natural settings. Thus, the complexity of the mutual mechanisms occurring in natural environment (e.g. interaction occurring between one and other microbes, plants and abiotic factors) (Duffy and Defago 1999; Droby et al. 2016) is lost in such screens. The newly introduced biocontrol strain has to survive in the plant environment, establish the stable population for extended time and produce factors important for disease control (Mazzola and Freilich 2017). It is also well established that the microbes persistence in the plant proximity or in soil environment depends not only on the attributes expressed by the microbial biocontrol agents themselves but the phenomenon is also determined by plant species or more specifically even by plant genotype (Berg et al. 2017).

The biocontrol potential of the mixture of bacterial or fungal or bacterial and fungal isolates, expressing distinct features important for control of (diverse) patho-gens, protection of plant and/or promotion the plant has been broadly studied (Duffy et al. 1996; Raupach and Kloepper 1998; Nandakumar et al. 2001; Domenech et al. 2006; Patel et al. 2018).

In the ideal situation, all members of the consortium originate from the same environment (e.g. they are associated with plant surface or plant roots), and as the

plants shape their microbiome (*via* root exudate, volatile organic compounds or other secondary metabolites) (Kumar et al. 2017; Compant et al. 2010), their performance should be more effective than the randomly selected consortia of isolates obtained from various environments. However, while creating the (artificial) microconsortia two aspects should be definitely considered: the compatibility of the biocontrol agents in the consortium and their collective performance on the plant (Thomloudi et al. 2019). Behavior of the entities within a consortium may trigger the efficacy of the consortium in environment. If a microorganism is inhibiting the growth of another member of the mixture, the protective potential of the consortium could be impaired due to the weakness of the entire consortium (e.g. overtaking of the population by its single member) (Niu et al. 2017) or could benefit from the increase of the plant protective or promoting attributes induced by particular member of the consortium (e.g. enhanced production of antimicrobials) (Tyc et al. 2014; des Essarts et al. 2016). The more, *in vitro* assays done for compatibility (which is defined as the lack of growth suppressive effect on each other within the consortium) of the strains in the consortium do not reflect the plant environment and aforementioned complexity of the biotic and abiotic stimuli. Regardless of whether single strain or the mixture of strains (intra- and inter- species or inter-domains) have been considered as the potential biocontrol agent(s), the same features should be verified within plant environment, as they facilitate the persistence of the BCAs and influence of plant fitness and plant resistance to the pathogens (Compant et al. 2010; Berg et al. 2017; Martínez-Hidalgo et al. 2018). These studies direct attention for selection of the microorganisms according to their capabilities and performance in plant environment as suggested by Köhl et al. (2011) or as perforemd by (Krzyzanowska et al. 2019).

It is now well-understood that plant microbiomes may serves as a source of valuable biocontrol agents (BCAs) (Handelsman and Stabb 1996; Mendes et al. 2011, 2013; Berg et al. 2017). These microbes may be used to control plant diseases both, as single strain compositions and/or as (artificial) microbial consortia (Spadaro and Droby 2016; Syed Ab Rahman et al. 2018; Thomloudi et al. 2019).

The new perspectives for a holistic approach to develop microbial consortia for agriculture could be based on the modern -omics technologies. The genome sequencing technology allows for rapid access to the genomic information, which in combination with bioinformatic tools (e.g. antiSMASH) may result in a fast discovery of novel antimicrobials produced by (plant beneficial) microbes (Medema et al. 2011; Cimermancic et al. 2014; Aleti et al. 2015; Krzyzanowska et al. 2016; Blin et al. 2017). The -omics approach (genomic/metagenomic, transcriptomic, proteomic and metabolomic) provides the massive data on the functional groups of microorganisms/genes/proteins or metabolites (Levy et al. 2018) that can be readily used to find new BCAs as well as new control mechanisms. Yet they also have limitations – still the question how to use wisely these data for the better understanding of plant-microbe interactions remains open.

12.3 Fundamental Research on Microbial Consortia Including Their Activity, Efficacy and Ecological Impact

In agriculture, biocontrol products may be applied on planting material (seeds, seedlings, cuttings, seed tubers, etc.), on roots, into the soil and/or on the foliar plant parts (Bashan et al. 2014). These plant-associated environments are recognized among the most complex systems on Earth (Raaijmakers et al. 2009). In such eco-logical habitats, introduced artificial microbial consortia may have advantages over the individual strains: they may express synergistic activity in establishing their presence in niche easier than applied individual strains, be able to broader the niche or even form effective metabolic networks supporting the activity of each member and hence the overall performance of the consortium (Sarma et al. 2015).

Due to the complexity of the natural and agricultural plant-associated systems and many variables influencing the final outcome, there is no an universal rationale for designing prospective artificial consortia for biological control (Johns et al. 2016). Combining microorganisms with already known and well characterized abil-ities and features seem to be the most straightforward approach to obtain effective synthetic microbial consortia (Kannan and Sureendar 2009). One thing to remain crucial is the evaluation of the mechanistic compatibility between consortium's members (Stockwell et al. 2011). Frequently, on the commercial scale, the members of the consortia are firstly only evaluated on the basis of their ability to inhibit the growth of the other members in the particular micro-consortium and only later ana-lyzed for their biocontrol activity towards a pathogen of interest. For example, *Pseudomonas fluorescens* A506, an active component of commercial product against fire blight named BlightBan A506 (NuFarm Americas, Burr Ridge, IL), pro-duces an extracellular protease which degrades antibiotic produced by *Pantoea vagans* strain C9-1 (Ishimaru et al. 1988), an active component of an another com-mercial product against the same disease, BlightBan C9-1 (NuFarm Americas) (Stockwell et al. 2011). In another approach, Raupach and Kloepper (1998) designed a mixture of *Bacillus pumilus* strain INR7, *Bacillus subtilis* strain GB03, and *Curtobacterium flaccumfaciens* strain ME1 of already proven biological control activities: strain INR7 (Waechter-Kristensen et al. 1994) and ME1 being able to induce systemic resistance (ISR) in cucumber, and strain GB03 expressing biocon-trol activity against *Rhizoctonia solani* and *Fusarium* spp. pathogens (Backman et al. 1998). The advantageous effect of combing together different strains lies within the increased consistency in effective control of the pathogens between experiments and overall better biocontrol activity when more than one pathogen are present on the same plant and/or field.

In order to avoid possible problems with the compatibility of the consortium's members, it is advised to use a collection of microorganisms and arrange them into

micro-consortia to test them in the target pathosystem. This approach was used to compare activity of nine *Pseudomonas* spp. strains against *Phytophthora infestans*. All 129 consortia containing two or three *Pseudomonas* spp. strains isolated from the potato rhizosphere or potato shoots were tested under laboratory conditions against the pathogen. The obtained results reviled that even though a strain S35 was the most efficient when applied alone, its protective effect was diminished when combined with other biocontrol strains in a consortium. Although the experiments did not lead to identification of several best candidates for commercialization, they showed that consortia containing bacterial isolates provide more consistent protective effect than individual antagonistic strains (Xu et al. 2011). Use of the combinations instead of single strains is quite an easy and straightforward approach also due to the fact that many laboratories possess a collection of pre-characterized strains isolated in the former studies that are ready to be screen in various combinations, environments and pathosystems (Príncipe et al. 2007; Bhattacharyya and Jha 2012; des Essarts et al. 2016).

Another possibility to design artificial microbial consortia is to obtain several microorganisms of different features collected from the same habitat (Príncipe et al. 2007; de Vrieze et al. 2018). The common origin of the biocontrol (antagonistic) strains should positively influence compatibility of the consortium. Furthermore, the different modes of antagonistic activities (e.g. production of plant hormones, nitrogen fixation, phosphate solubilization, antagonistic activity towards pathogens) should ensure effective protection of the plant health and fitness under environmental conditions and variable pathogen pressure (Kannan and Sureendar 2009).

Good examples of such approaches are three micro-consortia named Santalum (containing *Pseudomonas* sp. S1; *Bacillus* sp. S2; *Azotobacter* sp. S3; *Azospirillum* sp. S4; *Pseudomonas fluorescens* S5), Tamarindus (*Bacillus* sp. T1; *Pseudomonas* sp. T2; *Azotobacter vinelandii* T3; *Azospirillum* sp. T4; *Pseudomonas fluorescens* T5) and Ailanthus (*Aspergillu*s sp. A1; *Pseudomonas* sp. A2; *Azotobacter* sp. A3; *Azospirillum* sp. A4; *Pseudomonas fluorescens* A5). These three artificial microbial consortia are known to increase tomato resistance to fungal pathogens by activation of systemic acquired resistance in the plants (Kannan and Sureendar 2009).

It is well accepted now that the members of artificial microbial communities should preferably originated from exactly the same environment as the one in which there are designed to be used (Príncipe et al. 2007; des Essarts et al. 2016). This seems to be crucial also because bacterial population depends more on host organisms rather than soil type and therefore may not be able to perform in a rhizosphere of a different host plant (Bonito et al. 2014). Therefore, for example, Santhanam et al. (2015) used the bacterial and fungal isolates found in tobacco to fight sudden-wilt disease of the *Nicotiana attenuate*. This work led to the identification of a core consortium consisting of five strains: *Bacillus mojavensis* K1, *Pseudomonas frederiksbergensis* A176, *Arthrobacter nitroguajacolicus* E46, *Bacillus megaterium* B55 and *Pseudomonas azotoformans* A70 which when applied together was able to protect tobacco plant from sudden-wilt disease caused by *Alternaria* spp. (Santhanam et al. 2015).

Another way of designing the artificial microbial consortia for biological plant protection utilizes plants ability to recruit beneficial microorganisms when subjected to a biotic stresses (Berendsen et al. 2018). Soil suppressions are developed after the disease outbreak, suggesting active recruitment of plant beneficial microbes in order to fight the pathogens (Weller et al. 2002; Berendsen et al. 2018). Using this phenomenon, a consortium of three bacteria species has been developed, containing: *Xanthomonas* sp. WCS2014–23, *Stenotrophomonas* sp. WCS2014-113 and *Microbacterium* sp. WCS2014-259. These three species were actively recruited by *Arabidopsis thaliana* plants inoculated with a pathogen *Hyaloperonospora arabidopsidis*. Inoculation of healthy, pathogen-free Arabidopsis plants with the mentioned microbial consortium prior to infection significantly increased Arabidopsis resistance to *Hyaloperonospora arabidopsidis* (Berendsen et al. 2018).

Vital to remember, despite of the used strategy, is that the initial *in vitro* screening may not necessary result in a selection of a microbial consortium that will be active in the target (natural or agricultural or both) environments. Strains and/or consortia which are active *in vitro* may not show desired activity *in vivo* and *vice versa*. This inconsistency may be caused by several factors including difference in nutrient availability between natural environment and artificial media or the way how the isolates were screened for their biocontrol activity (Kamilova et al. 2006). For example, in the plant rhizosphere, bacteria from the genus *Collimonas* suppress pathogenic *Fusarium oxysporum* by competition for nutrients (Kamilova et al. 2007), yet they are unable to suppress the growth of this pathogen when grown together on artificial media under laboratory conditions. Another such an example is the mixture of four soil bacteria: *Brevundimonas* sp., *Luteibacter* sp., *Pedobacter* sp. and *Pseudomonas* sp. that possess antifungal activity towards *Rhizoctonia solani*, *Fusarium culmorum* and *Trichoderma harzianum*, while single bacterial strains do not or only weekly inhibit the growth of any of these pathogenic fungi *in vitro* (De Boer et al. 2007). In this example the presence of other antagonistic bacteria in the same environment (co-inoculation) triggers the production of secondary metabolites which, in turn, suppresses the growth of the pathogen.

It is now generally accepted that developing new effective microbial consortia for agricultural applications requires a better understanding of the plant – microbe interaction, particularly the knowledge on how plants recruit and assemble their beneficial microbiome is vital (Berendsen et al. 2018). Plants are colonized by non-random species of microorganisms, meaning the plant microbiome itself and the surrounding soil microbiome will be genetically dissimilar (Mendes et al. 2011). It seems that some bacterial genera are recruited by plants to be a part of the efficient beneficial microbiome more frequently than others. For example, *Bacillus* spp. (Ongena and Jacques 2008) and *Pseudomonas* spp. (Haas and Defago 2005) are commonly found when screened for antagonistic activity against plant pathogens and consequently used both in proof-of-concept experiments and commercial applications as biocontrol agents. But when comparing microbiomes of different specimens of *Ulva australis*, only a 15% similarity between the bacterial species is observed, as opposed to 70% similarity in terms of functional (genes and gene products) composition of the microorganisms (Burke et al. 2011). This suggests that

microbiomes are recruited rather based on the particular required function impor-tant for the community and/or host plant, not based on the microbial species. Microorganisms are selected from groups sharing functionalities (guilds, not neces-sarily composed of related organisms) according to the lottery (competition) model (Burke et al. 2011). In this model, in the plant rhizosphere many bacterial species compete for a small number of possible niches on, in and near the host plant and the one that arrives to the specific niche first and is able to survive can grow and win the competition (Chesson and Warner 1981).

The unsolved problem remains how the plant host recognizes the microorgan-isms possessing necessary (beneficial for plant fitness) functionalities. Plants recruit their microbiomes by producing root exudates, which makes the rhizosphere richer in nutrients in comparison to the surrounding root-less, bulk soil (Raaijmakers et al. 2009). Root exudates may attract various bacteria: some that can beneficial support-ing plant growth, but also accidently the pathogenic microbes (el Zahar Haichar et al. 2008). Pathogens feeding on the plant tissues have usually higher reproduction rate (due to life on the expanses of the plant) in comparison with the non-pathogenic (beneficial) microorganisms (leaving on secreted root exudates) and therefore they can overgrow the beneficial microbes. To be able to survive and compete in plant rhizosphere, plant beneficial bacteria are, in turn, more often resistant to a number of different microbial compounds (including antibiotics) (Wright 2010) and usually they are as well able to produce own antibiotics. The plant beneficial microorgan-isms are therefore promoted in more competitive environments in which the patho-gens may not be able to establish their populations efficiently (Scheuring and Yu 2012).

In plant environment, both pathogens and beneficial microorganisms can reach an equilibrium state where invasion of the new microbe from the outside would be virtually very difficult (Scheuring and Yu 2012). This steady state can be broken by a sudden change in the microbiome by so-called immigration rate (e.g. by adding biological control agents) or by the accessibility of the growth substrates (e.g. change in production of root exudates due to infection or mechanical damage, fer-tilization, drought). The plant microbiome may be therefore severely mechanisti-cally manipulated in order to promote plant beneficial microorganisms on the expanses of plant pathogens (Berendsen et al. 2018). The idea of creating environ-ment favoring a certain group of bacteria over some others is called screening (Archetti et al. 2011). In the screening approach no specific signal to promote some bacterial species or group of bacteria is used, but the environment itself selects the microorganisms for the function based on their ability to outcompete others (Archetti et al. 2011). According to the screening model, in order to ensure health of the host, the competitivity in the environment must be high and increasing over time (Scheuring and Yu 2012).

12.4 Successful Applications of Microbial Consortia-Based Biological Control

The market value of microbe-based bioproducts for agriculture is increasing annually (Arora et al. 2016b). The reasons behind it are diverse but generally fall into two main categories: firstly, the costs of production of chemical fertilizers is growing and, secondly, new legislations under which the use of chemical pesticides in agriculture should be limited are introduced in many countries worldwide (Pimentel 1991; Huffaker 2012). Moreover, the currently applied methods for managing some of the important plant pathogens are either inefficient or cost-ineffective, resulting in significant losses in agricultural productivity (Mehnaz 2016). All these issues create a need to find new, sustainable treatments to be applied in agriculture to improve plant health and crop yield (Arora et al. 2016a).

Despite the fact that the use of artificial microbial communities emerges as a promising alternative to the application of single plant beneficial strains, both in terms of plant growth promotion and the protection of plants against pathogens, the majority of commercially available bioproducts are based on a single bacterial species (Arora et al. 2016a). Only two companies, Marrone Bio Innovations (USA) and AgriLife (India), have registered and commercialized biocontrol products containing more than one microorganism (Table 12.1).

The small number of consortia-based biopesticides commercially used in agriculture is caused by the laborious and costly process of their registration (Arora et al. 2016b), especially in the European Union (Frederiks and Wesseler 2019). In case of microbial communities claimed to be biofertilizers, the registration process is simpler, resulting in a higher number of this type of bioproducts on the market (Lewis and Papavizas 1991). An interesting fact is that some bioproducts being commercialized as biofertilizers are known to have biocontrol properties. For example, Mapleton Agri Biotec Pty Limited gives information that its two biofertilizers: TwinN and Nitroguard containing microbial consortia have the ability to suppress *Fusarium* wilt but despite that, these products are not registered as disease control products (http://www.twinn.com.au). TwinNTM (Mapleton Agri Biotec Pty Limited) contains microbial consortium of Diazotrophs (>10^{11} cfu/vial) developed to promote plant growth by fixation of atmospheric nitrogen, production of plant growth stimulants (e.g. auxins), increasing nutrient availability (e.g. phosphate solubilization) and increasing soil health (e.g. by increasing the number of beneficial microbes, leading to decrease in root infection by *Fusarium*). TwinN is suited for application on broad acre crops including wheat, corn, barley, oats, sorghum, rice, cotton, soybean, lupins, mung beans, pastures and many others (http://www.twinn.com.au). NitroGuardTM (Mapleton Agri Biotec Pty Limited) contains microbial consortium of diazotrophs with *Bacillus* spp. (>10^{11} cfu/vial) developed to promote plant growth by fixation of atmospheric nitrogen, production of plant growth factors (e.g. auxins) increasing nutrient availability (e.g. phosphate solubilization) and increasing soil health (e.g. by increasing the number of beneficial microbes, leading to decrease in root infection by *Fusarium* spp.). NitroGuard is suited for broad acre

Table 12.1 Bioformulations based on microbial consortia registered as biocontrol products and available on the market (October 2019)

No.	Product	Producer	Composition	Target	Application	Reference
1	Bio-Tam 2.0TM	Marrone Bio Innovations	1–3% *Trichoderma asperellum* + 1–3% *Trichoderma gamsii*	Developed against soil-borne diseases caused by *Fusarium* spp., *Phytophthora* spp., *Pythium* spp., *Rhizoctonia* spp., *Sclerotinia* spp., *Sclerotium rolfsii*, *Thielaviopsis basicola, Verticillium* spp.	Suitable for application on most crops, including fruiting vegetables, leafy vegetables and cucurbits, and in greenhouse and field application	Preininger et al. (2018)
2	Biorub™	AgriLife	Microbial consortium 1 × 108 CFU/g. (spores, mycelial fragments and vegetative cell) composition not revealed	Developed against *Corticium salmonicolor* and *Erythricium salmonicolor*	Suitable for application on all types of crops. It can be applied as foliar spray, by soil application, and by stem swabbing	Preininger et al. (2018) http://www.agrilife.in
3	Biofit™	AgriLife	Microbial consortium (2% w/w) with an antifungal and plant growth promoting activity, composition not revealed	Developed against *Pythium, Alternaria, Xanthomonas, Rhizoctonia, Botrytis, Leveillula, Phakopsora, Sclerotium, Phytophthora, Peronospora, Sclerotinia Oidiopsis, Alternaria,* which cause diseases such as root rot, root wilt, seedling rot, early blight, late blight, leaf spot, stem rot and mildew diseases in crops, respectively.	Suitable for application on cereals, millets, pulses, oilseeds, fiber crops, sugar crops, forage crops, plantation crops, vegetables, fruits, spices, flowers, medicinal crops, aromatic crops, orchards and ornamentals	Mehnaz (2016) http://www.agrilife.in
4	Diebackcare™	AgriLife	Microbial consortium (2% w/w), composition not revealed	Developed against dieback disease of orchards	Suitable for application on orchards and should be applied on soil	Mehnaz (2016) http://www.agrilife.in

(continued)

Table 12.1 (continued)

No.	Product	Producer	Composition	Target	Application	Reference
5	Seedguard™	AgriLife	Microbial consortium (2% w/w) with antifungal properties which encourage seed germination and control seed-borne fungal disease, composition not revealed	Developed against most of fungal seed borne diseases	Suitable for application on cereals, millets, pulses, oilseeds, fiber crops, sugar crops, forage crops, plantation crops, vegetables, fruits, spices, flowers, medicinal crops, aromatic crops, orchards and ornamentals. It can be applied as a seed coating	Mehnaz (2016) http://www.agrilife.in
6	BorerGuard™	AgriLife	Microbial consortium (2% w/w) composition not revealed	Developed against egg plant shoot borer (*Leucinodes orbonalis*), tomato stalk borer (*Symmetrischema tangolias*), okra shoot borer, (*Earias vittela*), coffee white stem borer (*Xylotrechus quadripes*), grapes stem borer (*Celosterna scabrator*) maize stem borer (*Busseola fusca*), dark headed stem borer of rice (*Chilo polychysus*), gold-fringed stem borer (*Chilo auricilius*), pink stem borer, violet stem borer (Sesamia inferens), striped stem borer (*Chilo suppressalis*), white stem borer (*Scirpophaga innotata*), yellow stem borer (*Scirpophaga incertulas*), early stem borer of sugarcane (*Chilo infescatellus*), sugarcane top shoot borer (*Scirpophaga excerptalis*).	Suitable for application on egg plant, tomato, okra, coffee, grapes, rice, maize, sorghum and sugarcane	Mehnaz (2016) http://www.agrilife.in

crops including wheat, corn, barley, oats, sorghum, rice, cotton, soybean, lupins, mung beans, peanuts, pastures and many other crops. It is also used in several tree and vine crops including almonds, walnuts, pecan, apples, stone fruit, avocado, macadamia, citrus, blueberry, bananas and grapes (http://www.twinn.com.au).

There is undoubtedly a growing demand for biopesticides and biofertilizers on the market. The majority of companies producing bioproducts are cautious and do not share the information about the active components of their products as these can be copied and adopted by their competitors. The complexity, cost and long time necessary to release new bioproducts, especially in the European Union, has a large negative impact on the number and availability of the bioformulations on the marker. However, this situation is suspected to improve in the coming years thanks to the effort of international agricultural agencies (Arora et al. 2016b).

12.5 Considerations for Design of the Novel Synthetic Microbial Consortia for Biocontrol in Agriculture

Developing an efficient, marketable biocontrol agent is always a challenge, even with the help of guidelines found in the literature: for review see: (Köhl et al. 2011). At the same time, our expanding knowledge in the field of microbial ecology sug-gests that application of artificial microbial consortia in agriculture may have advan-tages over augmentation with single strains (Droby et al. 2016). The postulated advantages of microbial consortia include better colonization, broader spectrum of targeted plant pests or pathogens and higher reproducibility in expression of benefi-cial traits in the changing conditions (Fukui et al. 1999; Meyer and Roberts 2002; Droby et al. 2016). However, designing artificial consortia is difficult due to high complexity of possible microbe-microbe and plant-environment-microbe interactions.

A feature frequently mentioned in case of microbial consortia-based biocontrol agents is ‘strain compatibility’. The concept has its beginning in the metabolic links in the natural ecosystems, such as competition and cooperation, as well as in the fact that microorganisms can produce metabolites with antimicrobial features (Ghoul and Mitri 2016). By secreting antimicrobial compounds, the microorganisms are able to affect their fellow players on the consortium.

According to Swenson et al. (2000) (Swenson et al. 2000), an artificial microbial consortium can be assembled either by a ‘bottom up’ or a ‘top down’ approach. The ‘bottom up’ approach would involve using pre-obtained microbial strains (building blocks) and testing them in multiple combinations in order to identify an artificial consortium providing the desired outcome. Although simple in principle, this strat-egy is difficult to implement due to the high number of combinations to be initially tested. In practice, the majority of synthetic consortia reported so far to have biocon-trol purposes are combinations of strains that have been independently isolated and pre-selected based on certain properties, either *in vitro* or *in planta*, before

combining them into a new artificial consortium (Santhanam et al. 2015; Krzyzanowska et al. 2019). To some extent, the concept of random testing can be incorporated into this scenario. For example, Krzyzanowska and colleagues described the selection of a mixture of bacteria efficiently attenuating soft rot caused pathogens of genera *Pectobacterium* spp. and *Dickeya* spp. In this study, a pool of 22 strains with a reported *in vitro* or *in planta* antagonism towards at least one of the pathogens were randomly mixed and tested on potato tubers in storage, to result in a five-strain combination effective against a combination of *Pectobacterium* and *Dickeya* species most often associated with potato diseases in Europe (Krzyzanowska et al. 2019).

Second strategy to assemble a microbial consortia, referred to by Swenson and colleagues as the 'top down approach', involve testing many existing communities (microbiomes) for the desired outcome and then using them to generate next, more efficient generation of communities *via* adaptation strategy (Swenson et al. 2000). The resulting consortia are therefore mixtures of strains co-selected to achieve a certain well-defined goal. In plant sciences, this approach was successfully used to select consortia influencing the flowering time of *Arabidopsis thaliana* (Panke-Buisse et al. 2015) and comprising a minimal microbiome of maize, additionally showing protective properties against *Fusarium* (Niu et al. 2017).

To venture even further towards the evolution-based approach, a concept of co-breeding the plant and the microbiome was developed (Mueller and Sachs 2015). The strategy is not based on a use of a defined microbial-based product but rather on obtaining plant breeds that would recruit beneficial microbiomes from the environment and assuring a reservoir of 'functional' beneficial microbes in soil (Gopal and Gupta 2016; Raaijmakers and Mazzola 2016).

Microbial consortia designed for agriculture and resulting from the 'top down' approach may comprise a few strains (Panke-Buisse et al. 2015) but also be very complex or partially undefined. Therefore, the implementation of this concept to develop commercial agents for biocontrol would require not only a shift in how we view microbial plant protection products but, most of all, significant changes in the process of registration of biopesticides.

When designing a microbial consortium for commercial application, one needs to have in mind that a multi-strain biopesticide has to meet all the requirements for a single strain agent and more, therefore justifying the additional cost of its registration and manufacturing. Currently, there is still a need for statistically significant data to support the advantages of the application of microbial consortia over the use of the single strains in biocontrol. In a meta-analytic study, Xu et al. (2011) reported that actual synergy between co-applied strains is rare and, in many cases, the efficacy of combination of strains is either worse or not significantly better than of the best control agent from the mixture when applied alone (Xu et al. 2011). Moreover, experiments designed to investigate the presence or lack of protective effect often do not have enough statistical power to prove more subtle factors such decreased variance and increased reproducibility under variable experimental conditions Therefore, when designing and testing novel synthetic consortia for biocontrol, it is worth to take into account these aspects.

12.6 Future of Microbial Consortia-Based Biological Control in Agriculture

The future of bioproducts containing microbial consortia seems to be bright. The increasing knowledge on the beneficial effects of (artificial) microbial consortia for plant health and fitness will undoubtedly enable developments of new bioproducts for agriculture in the near future. This should not be a surprise taking into account that the most microorganisms under natural settings exist in consortia and form complex networks rather than occur alone. Advances in synthetic microbiology, microbial engineering and microbial ecology will furthermore enable development of 'simple' synthetic ecosystems supporting plant fitness and pathogen suppression which may have a direct impact on a way how the crops will be cultivated globally. It is worth to notice that in the future the design of microbial consortia for commercial purposes will be aided by synthetic biology. With the right omics data for the available microbial building blocks, advanced bioinformatic tools, sufficient understanding of the organizing principles and collective behavior of the microbial communities, as well as temporal and special dynamics within, a synthetic consortium providing a desired outcome could be designed *in silico* (Escalante et al. 2015; Agler et al. 2016; Lindemann et al. 2016; Vega and Gore 2018).

The biggest problem to be solved at the moment seems to be the fact that the current law concerning the use of (beneficial) individual microorganisms and/or microbial consortia in agriculture and food production is far behind the scientific developments in this field. Without the change in legislation, the commercialization of microbial consortia-based products will be delayed and will not happened as quickly as it is needed.

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3.3. Future perspectives

We can expect more and more biological control agent-based products to appear on the market, firstly thanks to the limitation of the usage of chemicals (Alabouvette et al., 2006), but also to the fact that some microbes can have multiple beneficial functions for the plant. They can protect plants from various diseases, increase their abiotic stress tolerance, and stimulate their growth (Amaresan et al., 2016). Especially the use of consortia seems to be a promising approach since they can increase the spectrum of activities and increase the consistency of activity (Denoth et al., 2002).

To find new promising strains for such applications, many researchers turn to comparative genomics to guide their research (Denoth et al., 2002). Based on identified plant beneficial strains, certain genetic features essential for their activity can be selected (Alavi et al., 2014; Dias et al., 2019) which later can be used to predict functionalities of newly isolated strains (De Vrieze et al., 2020).

The challenge will be to design compatible consortia. This could be achieved with the help of computational methods for designing consortia for industrial purposes (Bernstein & Carlson, 2012; Höffner & Barton, 2014; Rapp et al., 2020). We can expect that novel computational methods will aid the development of novel biocontrol agents, however, basic research will still be required to provide the data for the analysis.

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Academic Curriculum Vitae

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(2016 - 2022) Third cycle studies (**Doctora**l) „Life Sciences and Mathematics Interdisciplinary Doctoral Studies" Intercollegiate Faculty of Biotechnology at University of Gdansk and Medical University of Gdansk

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8-week practice in the mycological laboratory at University of Houston Downtown **US**

3-month practice in the Department of Mycorrhizal Symbioses at the Institute of Botany of the Czech Academy of Sciences **CZ**

1-month practice during field expedition of Bird Migration Research Station (University of Gdansk) in Barberspan Bird Sanctuary **ZA**

3-year employment in scientific project LIDER: "Usage of synergistic activity of lytic bacteriophages and antagonistic bacteria in biological protection of potato (Solanum tuberosum L.) from pectinolytic bacteria from Pectobacterium spp. and Dickeya spp. genera" project manager PhD Robert Czajkowski financed by NCBiR Intercollegiate Faculty of Biotechnology UG & MUG**PL**

7-month project manager "Investigation of colonization and survival on potato plant Surface by selected strains antagonistic towards potato pathogens from Dickeya and Pectobacterium genera" funded by Polish Ministry of Science and Higher Education Intercollegiate Faculty of Biotechnology UG & MUG **PL**

4-week training: "Training in genome and phenome analysis with dedicated software." Under the supervision of Prof. Paola Mattarelli in the Department of Agricultural and Food Sciences at University of Bologna, Bolonia, Italy, as beneficient of PROM program funded by Polish National Agency for Academic Exchange NAVA **IT**

7-month employment at Multidisciplinary Digital Publishing Institute MDPI Poland Sp. z.o.o. in the position of Assistant Editor for the International Journal of Molecular Sciences **PL**

Publications:

1. **Maciag, Tomasz**, Dorota M. Krzyzanowska, Lukasz Rabalski, Sylwia Jafra, and Robert Czajkowski. "Complete Genome Sequences of Five Gram-Negative Bacterial Strains Comprising Synthetic Bacterial Consortium "The Great Five" with Antagonistic Activity Against Plant-Pathogenic Pectobacterium spp. and Dickeya spp." Molecular Plant-Microbe Interactions (2022): MPMI-01.
2. Matuszewska, Marta, **Tomasz Maciąg**, Magdalena Rajewska, Aldona Wierzbicka, and Sylwia Jafra. "The carbon source-dependent pattern of antimicrobial activity and gene expression in Pseudomonas donghuensis P482." Scientific reports 11, no. 1 (2021): 1-17.
3. Czajkowski, Robert, Jakub Fikowicz-Krosko, **Tomasz Maciag**, Lukasz Rabalski, Paulina Czaplewska, Sylwia Jafra, Malwina Richert, Marta Krychowiak-Maśnicka, and Nicole Hugouvieux-Cotte-Pattat. "Genome-wide identification of Dickeya solani transcriptional units up-regulated in response to plant tissues from a crop-host Solanum tuberosum and a weed-host Solanum dulcamara." Frontiers in plant science 11 (2020): 1368.
4. **Maciag, Tomasz**, Dorota M. Krzyzanowska, Sylwia Jafra, Joanna Siwinska, and Robert Czajkowski. "The Great Five—an artificial bacterial consortium with antagonistic activity towards Pectobacterium spp. and Dickeya spp.: Formulation, shelf life, and the ability to prevent soft rot of potato in storage." Applied microbiology and biotechnology 104, no. 10 (2020): 4547-4561.
5. Czajkowski, Robert, **Tomasz Maciag,** Dorota M. Krzyzanowska, and Sylwia Jafra. "Biological Control Based on Microbial Consortia–From Theory to Commercial Products." In How Research Can Stimulate the Development of Commercial Biological Control Against Plant Diseases, pp. 183-202. Springer, Cham, 2020.
6. Krzyzanowska, Dorota M*., **Tomasz Maciag***, Joanna Siwinska, Marta Krychowiak, Sylwia Jafra, and Robert Czajkowski. "Compatible mixture of bacterial antagonists developed to protect potato tubers from Soft Rot caused by Pectobacterium spp. and Dickeya spp." Plant disease 103, no. 6 (2019): 1374-1382. **(*- contributed equally to this work)**
7. Krzyżanowska, Dorota M., Anna Supernat, **Tomasz Maciąg**, Marta Matuszewska, and Sylwia Jafra. "Selection of reference genes for measuring the expression of aiiO in Ochrobactrum quorumnocens A44 using RT-qPCR." Scientific reports 9, no. 1 (2019): 1-11.
8. Krzyżanowska, Dorota M., **Tomasz Maciąg**, Adam Ossowicki, Magdalena Rajewska, Zbigniew Kaczyński, Małgorzata Czerwicka, Łukasz Rąbalski, Paulina Czaplewska, and Sylwia Jafra. "Ochrobactrum quorumnocens sp. nov., a quorum quenching bacterium from the potato rhizosphere, and comparative genome analysis with related type strains." PLoS One 14, no. 1 (2019): e0210874.
9. Krzyżanowska, Dorota M., Adam Ossowicki, Magdalena Rajewska, **Tomasz Maciąg**, Magdalena Jabłońska, Michał Obuchowski, Stephan Heeb, and Sylwia Jafra. "When genome-based approach meets the "old but good": revealing genes involved in the antibacterial activity of Pseudomonas sp. P482 against soft rot pathogens." Frontiers in microbiology 7 (2016): 782.

Patents:

1. Robert Czajkowski, Dorota Krzyżanowska, **Tomasz Maciąg**, Sylwia Jafra Biopreparations based on microorganisms for protection of plants against bacterial infections to be used in agriculture P.431434, EP3495510
2. Robert Czajkowski, Dorota Krzyżanowska, **Tomasz Maciąg,** Sylwia Jafra, Joanna Siwińska Antagonistic bacterial strains, compostions thereof and use for plant protection P.423806 EP3495510
3. Robert Czajkowski, Dorota Krzyżanowska, **Tomasz Maciąg**, Sylwia Jafra, Joanna Siwińska Reagent for protecting microorganisms during lyophilization P.428215, pending, EP3670645 pending

Presentations:

1. **Tomasz Maciąg**, Sylwia Jafra , Robert Czajkowski „Antagonistic interaction between biocontrol strains, implications for biological plant control against soft rot disease" poster presentation on miCROPe conference „Microbe-assisted crop production – opportunities, challenges & needs" 4-7.12.2017 Vien, Austria
2. **Tomasz Maciąg**, Sylwia Jafra "Chemotaxis of Pseudomonas donguensis P482 towards root exudates" poster presentation on mikroBIOT conference the 4th Workshop on Microbiology in Health Care and Environmental Protection 19-21.09.2017 Łódź Poland
3. **Tomasz Maciąg,** Dorota Krzyżanowska, Joanna Siwińska, Sylwia Jafra, Robert Czajkowski "Bacteria and bacteriophages based biocontrol product against SRE in potato tubers" poster presentation on XIV Meeting of the Working Group Biological control of fungal and bacterial plant pathogens IOBC-WPRS 2016 Berlin Germany
4. **Tomasz Maciąg**, Magdalena Remisiewicz, Michał Redlisiak, Jarosław K. Nowakowski " Trends in populations of sellected species of passerines Passeriformes migrating throuh Operation Baltic stations between 1965-2012" II Meeting of Bird Ringers Gdańsk 18.11.18

Practical courses:

1. Biotechnology laboratory work in compliance with ISO17025 standards (30h) Intercollegiate Faculty of Biotechnology UG & MUG
2. Introduction to NGS data management and analysis (8h) ideas4biology Sp. z o.o.
3. Optimize your RNA workflow - hints and pitfalls of RT-qPCR (2h) Promega GmbH

Skills:

1. driving license
2. sailor's patent
3. English CAE certificate
4. MS Office
5. QGIS
6. R statistical software
7. autoclave operation course
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3. reading
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5. tabletop RPG
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Compatible mixture of bacterial antagonists developed to protect potato tubers from soft rot caused by *Pectobacterium* spp. and *Dickeya* spp [1]

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The Great Five—an artificial bacterial consortium with antagonistic activity towards *Pectobacterium* spp. and *Dickeya* spp.: formulation, shelf life, and the ability to prevent soft rot of potato in storage [1]

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Complete Genome Sequences of Five Gram-Negative Bacterial Strains Comprising Synthetic Bacterial Consortium "The Great Five" with Antagonistic Activity Against Plant-Pathogenic Pectobacterium spp. and Dickeya spp.[1]

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Biological Control Based on Microbial Consortia – From Theory to Commercial Products [1]

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[1] D. M. Krzyzanowska i T. Maciag dzielą równocenny wkład w powstanie

Polegał na:

1. Udziale w opracowaniu szczegółów podejścia metodycznego
2. Udziale w przygotowaniu i wykonaniu prac eksperymentalnych
3. Udziale w analizie i wizualizacji danych
4. Udziale w pisaniu manuskryptu

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Polegał na:

1. Udziale w przygotowaniu i wykonaniu prac eksperymentalnych
   Przygotowałem protokoły do przeprowadzonych eksperymentów, brałem czynny udział we większości przeprowadzonych eksperymentach i zbierałem i zestawiałem otrzymane dane
2. Udziale w analizie i wizualizacji danych
   Zbierałem wszystkie prezentowane dane, przeprowadziłem obliczenia i testy statystyczne. Przygotowałem pierwotne wersje wykresów

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Tomasz Maciąg

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Polegał na:

1. Udziale w przygotowaniu i wykonaniu prac eksperymentalnych

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[1] D. M. Krzyzanowska i T. Maciag dzielą równocenny wkład w powstanie

Polegał na:

1. Udziale w przygotowaniu i wykonaniu prac eksperymentalnych
   Ustaliłam wpływ badanych szczepów na przeżywalność *Caenorhabditis elegans*
2. Udziale w analizie i wizualizacji danych
   Zanalizowałam i zwizualizowałam dane z wpływu badanych szczepów na przeżywalność *Caenorhabditis elegans.*
3. Udziale w pozyskiwaniu środków
   Pozyskałam środki na badania z wykorzystaniem *Caenorhabditis elegans.*

Marta Krychowiak-Maśnicka

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[1] D. M. Krzyzanowska i T. Maciag dzielą równocenny wkład w powstanie

Polegał na:

1. współudziale w powstawaniu koncepcji badań
2. udziale w nadzorze merytorycznym nad projektem
3. udziale w pisaniu i edycji ostatecznej wersji manuskryptu.

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[1] D. M. Krzyzanowska i T. Maciag dzielą równocenny wkład w powstanie tej publikacji

Polegał na:

1. Pozyskaniu środków finansowanych do przeprowadzenia prac badawczych
2. Zarządzaniu projektem
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Polegał na:

1. Udziale w przygotowaniu i wykonaniu prac eksperymentalnych:
   Przygotowałem protokoły do przeprowadzonych eksperymentów, brałem czynny udział we wszystkich przeprowadzonych eksperymentach i zbierałem i zestawiałem otrzymane dane
2. Udziale w pisaniu pracy
   Przygotowałem pierwotną wersję manuskryptu, ze szczególnym wkładem w metody i wyniki
3. Udziale w analizie i wizualizacji danych
   Zbierałem wszystkie prezentowane dane, przeprowadziłem obliczenia i testy statystyczne. Przygotowałem pierwotne wersje wykresów

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Polegał na:

1. Udziale w opracowaniu szczegółów podejścia metodycznego
2. Udziale w przygotowaniu i wykonaniu prac eksperymentalnych:
3. Udziale w pisaniu pracy
4. Udziale w analizie i wizualizacji danych

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Polegał na:

1. współudziale w powstawaniu koncepcji badań
2. udziale w dyskusji wyników
3. udziale w pisaniu pracy

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Polegał na:

- Udziale w przygotowaniu i wykonaniu prac eksperymentalnych:
- Udziale w analizie i wizualizacji danych

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Polegał na:

1. Udziale w analizie i wizualizacji danych
   Wykonałem analizy porównawcze genomu i przygotowałem pierwotne wersje grafik
2. Przygotowaniu pierwotnej wersji manuskryptu

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Polegał na:

1. Udziale w analizie i wizualizacji danych
   Przygotowałam ostateczne wersje grafik
2. Udziale w pisaniu manuskryptu
   Brałam udział w poprawkach i przygotowaniu ostatecznej wersji manuskryptu

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Polegał na:

1. Udziale w analizie i wizualizacji danych

Wykonałem sekwencjonowanie genomów, złożenie i anotację.

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Polegał na:

1. Nadzór nad powstaniem rozdziału: Chapter 12: Biological Control Based on Microbial Consortia – From Theory to Commercial Products

2. Przygotowaniu Wstępu do rozdziału: Introduction

3. Przygotowaniu podrozdziału: Future of Microbial Consortia-Based Biological Control in Agriculture

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Polegał na:

1. Przygotowaniu pierwotnej wersji podrozdziału:
   Fundamental Research on Microbial Consortia Including Their Activity, Effcacy and Ecological Impact
2. Przygotowaniu pierwotnej wersji podrozdziału:
   Considerations for Design of the Novel Synthetic Microbial Consortia for Biocontrol in Agriculture
3. Udziale w przygotowaniu pierwotnej wersji podrozdziału:
   Successful Applications of Microbial Consortia-Based Biological Control

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   Successful Applications of Microbial Consortia-Based Biological Control

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Polegał na:

1. Przygotowanie podrozdziału:
   Unraveling the Mode of Action of Individual Biological Control Strains and Microbial Consortia

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