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Odziaływania społeczne pomiędzy bakteriami *Bacillus subtilis* i *Dickeya solani*

Social interactions between *Bacillus subtilis* and *Dickeya solani*

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Oświadczenie

Ja, niżej podpisana, oświadczam, że przedłożona praca dyplomowa została wykonana przeze mnie samodzielnie, nie narusza praw autorskich, interesów prawnych i materialnych innych osób.

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Abstract

Bacillus subtilis environmental isolates produce a large variety of compounds with antimicrobial activity. These bioactive metabolites have been regarded as a powerful weapon against many plant pathogens. To date, infection caused by the plant pathogen *Dickeya solani*, listed as one of the top ten bacterial pathogens of concern in agriculture, poses a significant challenge in crop management, underlined by the scarcity of effective treatments.

In this study, we investigated the environmental strain *B. subtilis* MB73/2 for its efficacy in controlling *D. solani* growth. To closely mimic the natural environment, we analysed the interaction between *B. subitlis* and *D. solani* on semi-solid agar, where bacteria can establish motility and social behaviours. Interestingly, inoculating both bacteria in the same swarming plate, the swarming of B. subtilis was interrupted at ~ 0.3 cm from the front of inoculation of *D. solani*, while the central colony of *Dickeya solani* was translocated entirely from the point of inoculation to the edge of the plate.

The presence of: (i) an inhibition zone between the interacting bacteria; (ii) a sharp front that *B. subtilis* is not capable of penetrating (iii) a coordinated and directional escaping of *D. solani*, led us to hypothesize that the observed interaction resembles a more complex antagonism than a simple avoidance mechanism.

Screening of single gene deletion mutants of *B. subtilis* and *D. solani*, led us to the conclusion that surfactin released by *B. subtilis* is required for, but not solely responsible for, *D. solani* escaping; while the Lys-R regulator in *Dickeya solani* is responsible for the inhibition zone.

Interactions between *B. subtilis* and *D. solani* described in this work are an example pf a pray-predator interaction in the context of bacterial communities. Obtained results clearly underscore the complexity of mechanisms underlying such phenomena which commonly occur in the nature.

Streszczenie

Izolaty środowiskowe *Bacillus subtilis* wytwarzają różnorodne związki o działaniu przeciwbakteryjnym. Uważa się, że te bioaktywne metabolity mogą stanowić ważną broń w zwalczaniu patogenów roślin. *Dickeya solani* jest bakterią wymienianą jako jeden z dziesięciu patogenów najbardziej istotnych dla rolnictwa. Infekcje tą bakterią stanowią znaczące wyzwanie dla uprawy roślin, szczególnie z uwagi na bardzo ograniczone możliwości jej eliminacji.

W niniejszej pracy zbadany został środowiskowy izolat *Bacillus subtilis* MB73/2 pod kątem jego skuteczności w kontrolowaniu wzrostu *D. solani*. Aby jak najwierniej odwzorować naturalne środowisko, interakcje pomiędzy *B. subtilis* i *D. solani* analizowane były na półstałym agarze, na którym bakterie te mogą wykazywać ruchliwość i zachowania społeczne. Co ciekawe, podczas wzrostu bakterii posianych na wspólnym podłożu przemieszczanie się *B. subtilis* było zahamowane około 0,3 cm przed punktem naniesienia *D. solani*, podczas gdy centralna kolonia *Dickeya solani* ulegała całościowemu przemieszczeniu w kierunku krawędzi płytki hodowlanej.

Obecność: (i) strefy zahamowania wzrostu pomiędzy oddziałującymi ze sobą bakteriami, (ii) ostrej granicy, której *B. subtilis* nie był w stanie przekroczyć, (iii) skoordynowanej i ukierunkowanej ucieczki *D. solani* skłoniła nas do postawienia hipotezy, iż zaobserwowane oddziaływanie przypomina złożony antagonizm bardziej, niż prosty mechanizm unikania.

Badanie efektów mutacji pojedynczych genów zarówno w *B. subtilis* jak i *D. solani* doprowadziły nas do wniosku, iż wydzielana przez *B. subtilis* surfaktyna jest niezbędna do ucieczki *D. solani*, niemniej jednak nie jest ona jedynym czynnikiem odpowiedzialnym za obserwowane zjawisko. Udało nam się natomiast wykazać, że za wytworzenie strefy zahamowania wzrostu odpowiada czynnik Lys-R *D. solani*.

Opisane w pracy oddziaływanie *B. subtilis* i *D. solani* stanowi przykład zjawiska interakcji drapieżnik-ofiara w odniesieniu do społeczności bakteryjnych. Uzyskane wyniki wyraźnie podkreślają złożoność mechanizmów odpowiedzialnych za tego typu zjawiska, które powszechnie występują w środowisku naturalnym.

I. INTRODUCTION

1 Soft Rot Pectobacteriaceae (SRP) and *Dickeya* sp.

Bacteria from the *Dickeya* genus are Gram-negative pectinolytic enterobacteria responsible for causing diseases in a wide range of crops, ornamentals, and environmental isolates from water. Together with bacteria from the genus *Pectobacterium*, they form the group of Soft Rot Enterobacteriaceae or Soft Rot Erwiniae (SRE). SRE were recently reclassified from the Enterobacteriaceae to the *Pectobacteriaceae* Family (Adeolu et al. 2016) and referred to as Soft rot Pectobacteriaceae (SRP). Therefore, according to the most recent classification, SRP belong to the kingdom of Bacteria, phylum Proteobacteria, class Gammaproteobacteria, order Enterobacteriales, family Pectobacteriaceae, and are divided into two genera, *Pectobacterium* and *Dickeya*. It is important to note that the order Enterobacteriales includes pathogens in humans and animals, such as the species *Escherichia coli*, *Salmonella enterica*, and *Yersinia pestis*, and economically significant phytopathogens such as members of the genera *Dickeya*, *Pectobacterium*, *Erwinia* and *Brenneria* (Adelou et al., 2016).

Although many bacteria are able to cause soft-rot diseases in plants, most studies have focused on the genetics and microbial ecology of *Pectobacterium* and *Dickeya*. These two genera use similar virulence strategies, have overlapping hosts and geographical distribution, and are often found together in the environment. According to the current knowledge, every identified species within the *Dickeya* and *Pectobacterium* genera has the potential to induce bacterial soft rot. However, the prevalence of this ability may be influenced by the methods researchers employ for pathogen isolation. Typically, *Dickeya* and *Pectobacterium* are isolated using semi-selective media containing pectate, and subsequent investigations focus on colonies that create pits on this medium. If there are *Dickeya* and *Pectobacterium* species that do not cause soft rot, it is probable that these would not be identified because of the isolation method. (Charkowski, 2018).

1.1 The *Dickeya* genus

The *Dickeya* genus has undergone major re-classification over the past decades. In 1917, all members of the *Enterobacteriaceae* were part of the genus *Erwinia*, which included both pectolytic (c.g. *Erwinia carotova* and *E. chrysanthemi*) and non-pectolytic (*E.*

amylovora) species (Winslow et al., 1917). The genus was named after one of the founders of phytobacteriology, Erwin Frink Smith.

In 1953, Burkholder et al. identified *Erwinia chrysanthemi*, named after its first isolation from *chrysanthemum*, as a member of the *Erwinia* genus. However, due to the wide range of plant hosts, in 1984 *E. chrysanthemi* was subdivided into six pathovars based on host specificity: *crysanthemi*, *dieffenbachia*, *dianthicola*, *paradisiaca*, *pathenii* and *zeae* (Lelliot and Dickey, 1984).

In 1998, all pectinolytic *erwiniae* were moved into the new genus of *Pectobacterium* based on their ability to produce pectinolytic enzymes. While *Pectobacterium astrosepticum* (Pa) and *P. carotovorum* subs. *carotovorum* (Pcc) remain within this genus; based on 16S rDNA, DNA-DNA hybridization, and biochemical characterization, *Pectobacterium* chrysanthemi (formerly *Erwinia chrysanthemi*) was assigned as the first species of the new genus *Dickeya*.

At the time of classification, the genus *Dickeya* included six species: *Dickeya chrysanthemi*, *Dickeya dadantii*, *Dickeya dianthicola*, *Dickeya* dieffenbachiae, *Dickeya paradisiaca*, and *Dickeya zeae* (Samson et al., 2005).

Members of this genus are motile, non-sporing, straight rod-shaped cell with rounded ends. The cells vary in size, ranging from 0.8 to 3.2 µm by 0.5 to 0.8 µm, and are equipped with numerous flagella (peritrichous) (Charkowsky, 2006). All these species comprise strains isolated from various plant hosts, including dicots and monocots, and do not appear to harbor real host specificity.

The classification of *Dickeya* species presents a formidable challenge when relying on phenotypic analyses, that are primarily based on biochemical and nutritional traits. Although this traditional approach remains valuable for the preliminary classification of numerous strains, the introduction of DNA sequencing techniques contributed to the re-classification of the genus. Mainly based on 16 rDNA comparisons, the *Dickeya* genus has evolved by identifying new *Dickeya* species and re-classifying others.

In 2012, *D. dieffenbachiae* was reclassified as a subspecies of *D. dadantii* (Brady et al., 2012). Then, *D. solani* was isolated for the first time in Poland in 2005 from symptomatic potato plants and identified as a new *Dickeya* species (Slawiak et al., 2009 and Potrykus et al., 2016). The recently discovered species *Dickeya fangzhongdai* was obtained from

pear trees in China (Tian et al., 2016) and orchids in various countries (Alic et al., 2018). On the other hand, certain members of the *D. zeae* species were reclassified, with rice strains now falling under *Dickeya oryzae* (Wang et al., 2020), and *Dickeya parazeae* being identified (Hugouvieux-Cotte-Pattat and Van Gijsegem, 2021). Three additional *Dickeya* species were isolated from water sources: *Dickeya aquatica* from freshwater in Scotland and Finland (Parkinson et al., 2014), *Dickeya lacustris* from lake water and the rhizosphere of waterside plants in France (Hugouvieux-Cotte-Pattat et al., 2019), and *Dickeya undicola* from water samples in Malaysia and France (Oulghazi et al., 2019). Moreover, strains from a collection in Australia, initially isolated from sugarcane were re-classified as *D. poaceiphila* (Hugouvieux-Cotte-Pattat et al., 2020). Lastly. the species *D. paradisiaca* has recently been reclassified under the new genus *Musicola* (Hugouvieux-Cotte-Pattat et al., 2021).

Thus, the genus currently encounters twelve recognized species: *D. aquatica*, *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. fangzhongdai*, *D. lacustris*, *D. oryzae*, *D. paradisiaca*, *D. poaceiphila*, *D. solani*, *D. undicola*, and *D. zeae*. Based on phylogenetic analysis, *D. dadantii*, *D. dianthicola*, *D. fangzhongdai*, *D. solani*, and *D. undicola* have been grouped in the "main *Dickeya* clade." This group shares high ANI values (89 to 94%) and more than 50% of their protein families, including virulence genes and at least ten pectate lyases (Hugouvieux-Cotte-Pattat et al., 2023).

1.2 *Dickeya solani* – the fast-spreading potato-killer

Until 2000, potato blackleg infections in Europe were caused mainly by *D. dianthicola* and *P. atrosepticum* (Perombelon, 2002). However, over the past two decades, we witnessed a radical shift: the incidence of seed potato infections caused by *Dickeya sp.* has risen, surpassing those induced by *Pectobacterium* sp. (Toth et al., 2011). Moreover, all European potato isolates of *Dickeya* tested appeared to be *D. dianthicola* until 2004 when researchers across Europe identified the new biovar-3 *Dickeya* sp.

This novel clade was linked to the increase of severe blackleg disease in Europe. The biovar-3 *Dickeya* sp appeared different from the known *Dickeya* species (Toth et al., 2011). Despite a DNA-DNA hybridization (DDH) experiment revealing a substantial 72% relatedness between the type strains of the biovar-3 *Dickeya sp.* and *D. dadantii*, surpassing the species delineation threshold, conflicting results emerged from the pairwise Average Nucleotide Identity (ANI) calculation. With a value of 0.94, it leaned

towards favouring the separation of these two taxa. Consequently, these atypical strains were officially described as a novel species in 2014 under the name of *D. solani* (Van Der Wolf et al., 2014).

D. solani is currently the predominant bacterial potato pathogen in Europe, and it is recognized as the causal agent of the potato blackleg outbreak in Europe in the 2000s. The new pathogen has replaced other phytopathogens: up to 25% of the potato blackleg incidences in the Netherlands, Belgium and France are caused by *D. solani* (Toth et al. 2011).

D. solani strains are considered to be highly aggressive; they possess a wider optimal temperature range for disease development and require lower inoculum levels for infection spread. Moreover, they seem to be able to colonize the roots of potato plants and spread through the plants' vascular system more efficiently (Toth et al., 2011; Czajkowski et al., 2013). Field studies spanning three years in the Netherlands involving *D. dianthicola* and *D. solani* revealed annual disease detection variations among strains. The conclusions drawn by Czajkowski and colleagues (2011) suggest that *D. solani*'s features enable more efficient plant colonization than those of *D. dianthicola*. This heightened efficiency might explain why *D. solani* outcompeted other *Dickeya* species. Further investigations have elucidated that *D. solani*'s competitive advantage is particularly pronounced at higher temperatures (28° C), which holds significant implications for the increased importance of this pathogen in response to global warming (Czajkowski et al., 2011). Furthermore, *D. solani* has been suggested to be less susceptible to antimicrobial metabolites produced by saprophytic bacteria associated within the potato ecosystem. Due to the fast-spreading and high virulence, *Dickeya solani* has been included in the top 10 phytopathogens of interest in agriculture (Mansfield et al., 2012).

Compared to other *Dickeya* species, *D. solani* has a very narrow host range; most strains have been isolated from infected potatoes, with only one strain isolated from the ornamental muscari, two strains isolated from hyacinth, and two strains isolated from water (Toth et al., 2011). Moreover, analysed strains from different geographical regions and hosts were consistently recovered as a homogeneous cluster, suggesting a clonal origin (Sławiak et al., 2009; Van der Wolf et al., 2014).

The clonal origin and reduced host range strengthen the hypothesis that *D. solani* has spread to potatoes from infected ornamental plants, possibly via irrigation water and seed trade. While the import of potatoes into Europe is strictly controlled, the import of ornamentals is less closely regulated with the entry of millions of plants for planting (Czajkowski et al., 2013). For instance, *D. solani* strains isolated from potatoes in multiple countries were found to be closely related to a Dutch strain isolated from hyacinth bulbs (Parkinson et al. 2014).

Since the description of *D. solani*, the pathogen has been detected within European territories and other continents. Phylogenetic analyses on strains from culture collections have revealed the establishment of *D. solani* in Switzerland as early as in 1990s (Pédron et al., 2021), long before this species caused the major outbreak that Europe faced later in the 2000s. The new strain spread in Europe in less than 5 years through the trade of infected seed potatoes from the Netherlands (Toth et al., 2011). It likely entered Israel in 2004 through infected seed potatoes imported from the Netherlands, subsequently being intercepted in exported seed potatoes from France in 2009 and Germany (Tsror et al., 2009). The first report of *D. solani* in Poland dates back to 2005 (Slawiak et al., 2009), possibly introduced through imported potatoes from the Netherlands. Extensive surveys in Polish seed potato fields and water sources between 2009 and 2013 revealed the pathogen's presence, with varying intensity depending on climatic conditions (Potrykus et al., 2016). In Norway, *D. solani* was initially reported in 2012 in potatoes grown from imported seed. The pathogen's likely first isolation in Spain occurred in Valencia in 2002 (Palacio-Bielsa et al., 2006). Its detection in potatoes of Swedish, German, and Finnish origin indicates its establishment in diverse production systems (Rölin and Nilsson, 2011). Finland noted its first occurrence in 2004, with the highest incidence observed in 2006 (Degefu et al., 2013). In Crete, Greece, *D. solani* was first recorded in 2009. The pathogen made its debut in England and Wales in 2007 and in Scotland in 2009 (Cahill et al., 2010). Despite a monitoring program in Scotland since 2006, seed potatoes of Scottish origin were reported to be free from *Dickeya sp.* in 2010; however, the bacterium was found in potatoes entering Scotland for processing and planting (Cahill et al., 2010). Since 2005, *D. solani* has become the predominant cause of blackleg in Belgium (ILVO, 2010), and it significantly impacts blackleg in Switzerland (de Werra et al., 2020). In Georgia, the pathogen was first confirmed in 2008, likely originating from imported seed potatoes from the Netherlands and Germany (Tsror et al., 2017). Turkey reported its first detection

of *D. solani* in 2016 (Ozturk and Aksoy, 2017). Furthermore, *D. solani* was identified in the healthy potato rhizosphere in Germany in 2006 (Potrykus et al., 2014). In 2013, blackleg of potatoes was observed in a commercial field in Minas Gerais, Brazil, with the pathogen identified as *D. solani*, marking one of the initial reports in the New World (Cardoza et al., 2017).

1.3 Economic impact of *Dickeya solani* and other SRP

Dickeya solani, has emerged as a significant threat to potato (Solanum tuberosum) production globally. Potato ranks as the fourth most important food crop in the world after maize, wheat, and rice in terms of human consumption, and it is recommended as a food security crop by the United Nations (FAO, 2019).

At present, the global standard for potato production is 376 million tons on an estimated 19 million hectares of farmland worldwide, with one-third of the world's potatoes produced in China and India (Statista, Global potato production 2001-202, https://www.statista.com). The potato yield in the EU was 55.3 million tonnes in 2020, with Germany leading in production, followed by Poland, France, Netherlands, Belgium, and the UK (Fig. 1) (Eurostat, 2021).

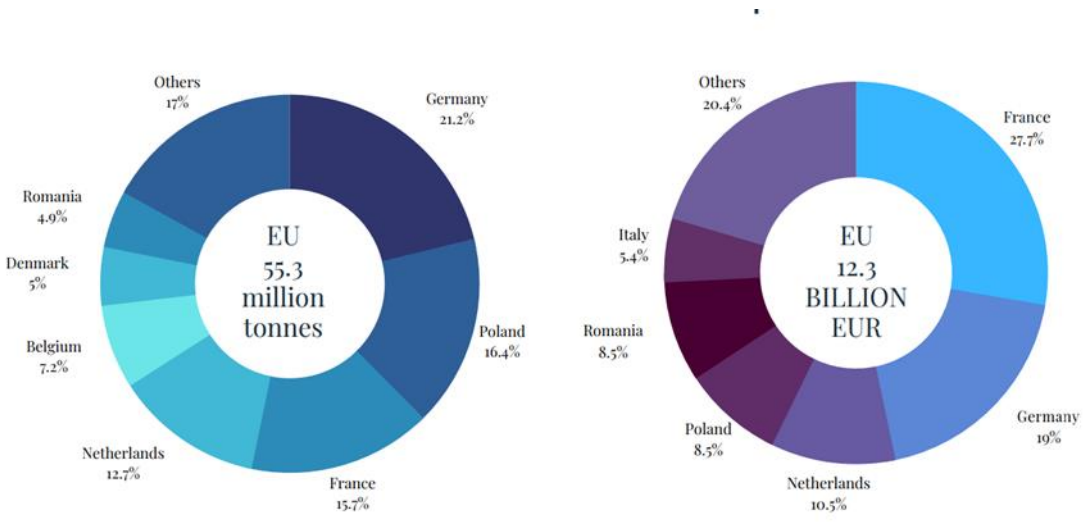

Figure 1- A - Potato yield in Europe by country; B - Potato revenue in EU by country. Data source: Statista, Global potato production 2021

The market value of potatoes in the EU was estimated at EUR 12.3 billion, representing 3.1% of the total EU agriculture production. Due to the concentration of major potato production in a few countries, the potato trade has become a significant and substantial market. In 2020, Member States engaged in extensive intra-EU trade, amounting to

approximately 7.0 million tonnes of potatoes. Notably, the European Union (EU) is a net exporter, trading 1.4 million tonnes of potatoes, valued at EUR 509 million (Statista, 2021).

Quantitative, standardized information on crop losses is difficult to compile and compare across crops, agroecosystems, and regions. As a result, information is usually retrieved indirectly. The economic impact of Dickey sp. infection on potato cultivation is hard to estimate as symptoms caused by *Dickeya sp.* and other SRP are impossible to differentiate. Consequently, researchers frequently turn to assessing the broader economic impact of SRP.

SRP infections are among the most important diseases for seed and ware potato production. Globally, losses in the potato production system are estimated at 16% on average (Oerke, 2012). In the EU, overall loss in the potato sector is estimated at EUR 46M with very high variability over the years. In Switzerland, losses caused by SRP are mostly dependent on the downgrading (9.8%) and rejection during seed certification (40.7%) (Dupuis et al., 2021). Blackleg causes rejection of the seed during seed inspection of around 25-45% in France. The Netherlands has estimated that costs for downgraded or rejected seeds reached EUR 12M per year (Dupuis et al., 2021).

In EU the European Plant Protection Organization (EPPO, https://www.eppo.int) has introduced a common certification for the marketing of seed potato; this is done to ensure that the seed potatoes meet the minimum EU requirements for quality and health (Directive 2002/56/EC). There are different certification classes corresponding to the years of multiplication and quality of the seed. *Dickeya sp.* were listed as A2 quarantine pests in 1982, 1988, and 1990 with a zero-tolerance scheme (Toth et al., 2011). Scotlans is the only country within the EU that still adopts a zero-tollerance of *Dickeya solani* infected seed, as all seeds are tested for *Dickeya sp.* before planting. (CABI, 2022; https://www.cabidigitallibrary.org). According to the EU certification scheme, SRP are currently listed among the RNQPs (Regulated Non-Quarantine Pests) that are allowed up to a defined threshold. When the incidence of blackleg falls short of meeting the minimum certification standards, the affected lot faces rejection and the potatoes are relegated to sale exclusively as ware. In cases where the percentage of blackleg symptoms observed during field inspection surpasses the acceptable limit for a certification class but remains below the rejection threshold, the seed lot undergoes downgrading to a lower class,

subject to the corresponding minimum requirements (EPPO, 2017). For instance, Swiss regulations stipulate stringent criteria for the rejection of potato lots based on blackleg incidence. Specifically, no blackleg is permitted in fields of pre-basic seed and in the initial basic seed class. However, a tolerance of 0.02% for blackleg is permissible in basic classes, while the last class of basic seed allows for a slightly higher tolerance of 0.1%. In the ultimate stage of seed multiplication, the A class (certified seed) permits up to 1% blackleg incidence (ordinance 916.151.1).

SRP are also the major cause of yield reduction in potato production. The prevalence of blackleg in the field directly correlates with the extent of yield loss. This conclusion aligns with findings from Israel, where it has been established that a disease incidence of 15% or higher results in substantial yield losses, reaching up to 56% (Tsror and Zig, 2017). Moreover, additional costs are linked to the roguing of infected or simply atypical potatoes. These plants need to be removed from the field before the inspection and represent an additional cost for the grower (Dupuis et al., 2021)

SRP can penetrate the vascular system, affect plant transpiration, and induce partial blocking of the vessels. Therefore, even if the plant does not show symptoms, they can induce soft rot on the progeny, especially when tubers are harvested in wet conditions (Czajkowski et al., 2010). Since there is zero tolerance for rotting tubers directed to industry or fresh market (Directive 2002/56/EC), the development of soft-rot is responsible for post-harvest losses.

In conclusion, financial losses attributed to SRP are mainly caused by yield reduction and seed rejection and downgrading. Addition costs to the grower are attributed to the roguing, seed replacement and post-harvest rejection due to soft rot. Due to the lack of data collection over the years and in different countries, it is hard to translate these costs into numbers. Researchers tried to recalculate the losses based on the Swiss data published by Prins and Breukers in 2008. The losses were retrieved indirectly using data comparison between: revenues (income from potato production); gross margin (difference between revenues and costs) and the total area dedicated to the European potato sector (ha per seed potatoes, table potatoes and processing potatoes). Based on the collected data, the overall loss for the entire sector was estimated at EUR 45.6M for 2002 (Dupuis et al., 2021).

1.4 Management and control strategies for infections caused by SRP

The traditional approach to controlling soft rot and blackleg in potatoes in the EU relies on the certification system and seed classification previously described. In general, potato seed production typically begins with pathogen-tested nuclear stock microplants, and field production is constrained to a limited number of generations to prevent the accumulation of pathogens, including *D. solani* (Toth et al., 2011).

Dickeya is predominantly seed-borne; it has been widely demonstrated that the infected seed (mother) tubers represent the main source of spreading. Significant efforts are directed toward minimizing the risk of contamination. One such approach involves the use of true seeds, generated from sexual crosses, which are considered free from blackleg bacteria. This method offers advantages such as ease of large-scale production and not requiring cold storage facilities. However, it comes with the drawback of genetic diversity, necessitating meticulous selection in each generation for desirable traits (Czajkowski et al., 2011).

Mechanical harvesting, likely to increase tuber-to-tuber spreading, is avoided during the early stages of pre-basic seed multiplication. Control measures also include cleaning and disinfection of machinery and equipment.

A series of studies spanning different countries, such as Australia, Sweden, the Netherlands, Finland, and Scotland, sound the alarm on the potential contamination of irrigation water with *Dickeya sp.* (Toth et al., 2011). This underscores a looming risk of waterborne transmission, prompting the consideration of practical measures such as restricting irrigation from these sources.

It's crucial to emphasize that the primary line of defense against *Dickeya sp.* infection lies in methods of avoiding contamination. Once this stealthy pathogen infiltrates the xylem or lenticels of the plant, effective treatment methods become an elusive quest, rendering infected plants beyond the reach of a cure.

1.4.1 Physical and chemical treatments

Physical and chemical control methods of seed tuber show promise in reducing SRP incidence; however, careful consideration of potential side effects, environmental impact,

and practicality is essential for developing sustainable blackleg and soft rot management strategies.

Dickeya sp. are vascular pathogens with the capacity to infect potato tubers internally. Given their ability to reside within the inner tissues of tubers, superficial treatments are largely ineffective. Consequently, traditional physical measures and chemical control agents may not significantly contribute to the control of SRP. Although physical control methods are environmentally friendly, they have limited success on bacteria residing in the inner of the plant and can harm the tuber. On the other hand, chemical treatments, while potentially more effective, introduce environmental risks. The use of antibiotics, for example, raises concerns about contributing to antibiotic resistance.

The table below (Tab.) summarizes the most common physical and chemical seed treatments and their limitations.

Table 1-Physical and Chemical treatments in the management of SRP infections

Treatment	Advantages	Limitations	Reference
Physical treatments			
Hot water	Hot water treatment at 55°C for 5 minutes, followed by drying, demonstrated effective blackleg control.	Failure to dry can result in multiplication of survived bacteria and rotting.	Pérombelon et al., 1989.
Steam	Steam treatment reduced tuber periderm infection from 26–59% to 1–3%.	Tuber physiology can be altered; treatment can affect beneficial organisms.	Afek and Orenstein, 2002
Hot Dry Air	Hot dry air at 50°C eliminated external *Pectobacterium* sp. populations without hindering tuber sprouting.	Inability to kill bacteria located inside the tuber.s	Bartz and Kelman, 1986
UV radiation	UV radiation showed effectiveness in eliminating superficial contamination.	Not suitable for high throughput applications.	Ranganna et al., 1997
Chemical treatments			
Antibiotics	Antibiotics, such as streptomycin and its derivatives, are highly effective against SRP.	High risk of introducing antibiotic resistance in pathogens of humans or animals.	Bonde and de Souza 1953, Czajkowski et al., 2011
Chemical disinfectants	1% sodium hypochlorite and 1% MennoClean	Associated phytotoxicity. MennoClean emerged as	Czajkowski et al., 2014

	water solutions demonstrated effectiveness in killing the pathogen, reducing population densities on tuber surfaces, and minimizing tuber soft rot incidences with relatively low phytotoxicity.	a potentially better candidate than sodium hypochlorite due to lower toxicity. Should be further investigated in fields	
Essential oils	Thyme oil showed high antibacterial activity; potential for wider application pending further research.	Further investigation is needed for tuber treatments and potential side effects.	Cai et al., 2022
Organic and Inorganic salts	Cationic ions released from organic and inorganic salts can inhibit the growth of SRP in vitro. Some of them are already approved as food preservatives.	Potential phytotoxicity	Mills et al., 2006
AgNPS, Silver nanoparticles	Silver nanoparticles stabilized by pectin or sodium dodecyl sulfate demonstrated bactericidal activity.	Further investigation is needed. Not tested in fields.	Dzimitrowicz et al., 2018; Hossain et al., 2019

1.4.2 Role of calcium and nitrogen in plant resistance

Plant nutrition is a crucial determinant of natural disease resistance, influencing plant growth, interactions with pathogens, and overall fitness status (McGovern et al., 1985). Deficiencies in essential elements often render plants more susceptible to diseases. Among these essential elements, calcium emerges as a key player in plant resistance against bacterial pathogens (Bateman and Millar, 1966 reviewed in 2003). Calcium ions enhance the structure and integrity of plant cell wall components, fortifying resistance to diseases involving tissue maceration. Calcium fertilization has demonstrated its efficacy in reducing soft rot caused by *Pectobacterium* sp. in various crops, such as Chinese cabbage and beans (Platero and Tejerina, 2008). Nitrogen levels also impact susceptibility to soft rot pathogens, as demonstrated in *Philodendron selloum* affected by *Dickeya sp.* (Haygood et al., 1982). While the effect of nitrogen levels on blackleg and soft rot in potatoes requires further exploration, Graham and Harper (1966) observed lower blackleg incidence with higher nitrogen fertilizer levels.

A balanced fertilization approach and increased calcium content in soils, while not standalone solutions, can form part of an integrated control strategy against blackleg and soft rot pathogens.

1.4.3 Breeding for resistance

Breeding for resistance against blackleg and soft rot caused by *Dickeya* and *Pectobacterium* species in potatoes has been challenging, with no commercial cultivars demonstrating complete immunity. While some cultivars exhibit partial resistance, breeding endeavors have faced only partial success in enhancing this resistance. Currently, there is no documented evidence supporting the existence of species or strain-specific resistance in potatoes against soft rot bacteria. Moreover, gene-for-gene resistance remains unobserved in the context of *Dickeya sp.*, and the mechanisms underlying resistance to *Dickeya sp.* in wild potato species are not well elucidated, as highlighted by Charkowski et al. (2020).

S. chacoense M6 was found to be resistant to *Pectobacterium* (Leisner et al. 2018). However, the resistance mechanism has not been fully elucidated due to the complexity of the plant secretion system. The plant extract, despite lacking antimicrobial activity, displayed a high concentration of phenolic compounds. This observation prompted the researcher to hypothesize that resistance might be attributed to quorum quenching. (Joshi et al., 2021).

In 2015, Poland registered the breeding cultivar Mieszko, which showed a higher level of partial resistance to *D. solani* than 10 other potato cultivars (Lebecka et al., 2021). The authors attempted to elucidate the mechanism of plant resistance to *Dickeya solani* and tried to identify QTLs (quantitative trait loci) for potato tuber resistance. Tubers were evaluated for resistance severity to and incidence of *D. solani*. The study pinpointed significant QTLs for disease severity and incidence in overlapping regions on potato chromosome IV, with an additional QTL for disease severity located on chromosome II. Although limited, this study can contribute to define candidate genes and marker development in potato breeding programs.

In the ongoing pursuit of solutions, modern breeding techniques, including transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced palindromic repeats/CRISPR-associated 9 (CRISPR/Cas9), have emerged as precise tools for developing potato varieties resistant to pathogens (Hameed et al., 2018). The

effectiveness of CRISPR/Cas9 in conferring resistance against plant pathogenic bacteria, as demonstrated in various crops (Jia et al., 2017), holds significant promise for addressing the longstanding challenges in potato breeding. However, the widespread adoption and commercial success of these genetically modified crops face hurdles related to public and governmental acceptance.

To address concerns surrounding the acceptance of genetically modified crops, a potential solution lies in the adoption of a cisgenic approach. In this approach, the genetic modification introduces a natural gene from a crossable sexually compatible plant into the recipient plant, providing an alternative to the transgenic methods employed in the early days of biotechnology (Schouten et al., 2006). This nuanced strategy holds the potential to alleviate objections and foster a more receptive environment for the development and utilization of genetically modified potato crops resistant to bacterial pathogens (Charkowski et al., 2020).

1.4.4 Treatment with elicitors: inducing plant defence response

Exploring elicitors as a plant defence mechanism against bacterial infections provides a promising alternative to traditional chemical and physical treatments. Plants have evolved various defence mechanisms to combat bacterial pathogens, activating specific responses upon infection. Induced resistance (IR) encompasses induced systemic resistance (ISR) and systemic acquired resistance (SAR), both leading to phenotypic responses against pathogens (Vallad and Goodman 2004). SAR, primarily induced by abiotic and biotic elicitors, relies on the salicylic acid (SA) signal molecule. While ISR is mediated by plant-growth-promoting rhizosphere bacteria and involves ethylene and jasmonic acid, SAR depends on exposure to elicitors and is associated with SA. Chemicals like SA, benzothiadiazole (BTH), and others can activate SAR without direct pathogen interaction (Czajkowski et al., 2014)

The efficacy of SA-mediated response was demonstrated in vitro on *D. solani* by Czajkowski et al., 2014. Application of SA prevented bacteria from colonizing the plants with an efficacy of 100%, although the resistance was found dose-dependent.

Despite encouraging results, the practical application of salicylic acid (SA) in controlling potato blackleg caused by *Dickeya solani* requires further research. To translate promising results into viable and effective strategies for potato blackleg management, several crucial factors need thorough investigation, including: optimal application conditions, cultivar-

specific responses, response to other pathogens, environmental impact and the molecular mechanism of protection (Czajkowski et al., 2020)

1.4.5 Biocontrol

As previously described, the management of soft rot and blackleg diseases caused by SRP poses significant challenges, primarily due to ineffective control strategies. Traditional methods, such as chemical and physical treatments, have proven inadequate, and the lack of crop varieties resistant to these diseases further complicates the scenario.

In line with the global intention to reduce the use of pesticides in agriculture, in 2020, the Farm to Fork Strategy by the European Commission introduced two pesticide reduction targets: a 50% reduction in the use and risk of chemical pesticides and a 50% reduction in the use of more hazardous pesticides. This commitment has resulted in a measurable impact, as evidenced by the global decline in crop protection chemical usage from 2.75 to 2.66 million metric tons between 2017 and 2021 (Statista, 2021). Therefore, in the last decades, a lot of effort has been devoted to developing new strategies, and biocontrol stands out as a promising and viable alternative, aligning with the sustainable agriculture goal.

Microbial control agents (MCAs) include bacteria, fungi, and bacteriophages that are able to control pests and diseases (FAO; www.fao.org). Over the last decades, several attempts have been made to identify MCAs with some activity against SRP. Initial identification of potential biocontrol species was based on the random isolation of bacteria from the potato rhizosphere or periderm that showed some antagonistic activity against SRP *in vitro*. The soil, rhizosphere, and endophytic microbiomes are recognized as rich sources of microbial communities that may possess advantageous attributes for enhancing plant resistance against various pathogens (Berendsen et al., 2012). These isolates are mainly classified into the genera *Bacillus*, *Pseudomonas*, and *Serratia*.

Microbial antagonists operate their disease control through different mechanisms mainly divided into two categories: those that involve direct interaction between the two antagonists and those independent from physical contact (Vero et al., 2023)

Some MCAs exert their action only when they are in close proximity and contact with their antagonists. For instance, their cell-free supernatant (CFS) demonstrates no inhibitory effect on the pathogen. This is the case of bacteria predators. Some bacteria

behave as microbial predators, killing other bacteria. *Bdellovibrio* and-like organisms (BALOs) exhibit efficient predation on *D. solani* when co-cultivated with the phytopathogen up to the complete prevention of the disease (Youdkes et al., 2020). However, the main limitation of this biological control method is its strict dependence on the simultaneous presence of both populations, which poses challenges in effectively managing this approach in field applications.

Most Microbial Antagonistic Agents (MCAs) exert their biocontrol activities primarily by either indirectly outcompeting pathogens for resources or directly secreting toxins and other metabolites with bactericidal or bacteriostatic action (Vero et al., 2023).

Bacteria can produce primary metabolites such as ethanol, lactic and acetic acid that inhibit the growth of other bacteria. Lactic acid, produced by *Lactoacillus farciminis*, demonstrates the capacity to suppress soft rot diseases caused by SRP. The Cell-Free Supernatant (CFS) exhibited robust antibacterial activity in vivo, resulting in the eradication of 99.2% of pathogens upon treatment with CFS on pepper (Li et al., 2024).

Most antimicrobial compounds are secondary metabolites secreted during the stationary phase of growth. Several species of *Bacillus* and *PaeniBacillus* can produce lipopeptides and glycolipids with antimicrobial activity against SRP (Sharga and Lyon., 1998; Cladera-Olivera et al., 2006; Azaiez et al., 2018; Hossain et al., 2023). These amphiphilic molecules damage the cell membranes and facilitate the plasmolysis. However, it is worth noting that some *Bacillus sp.* have also been shown to cause severe damage to potato wounds while conferring protection against SRP (Zaho et al., 2013).

Bacteria also produce VOCs (volatile organic compounds) that inhibit the growth of plant pathogens and represent an important biological mechanism for controlling plant diseases (Vero et al., 2023). VOCs produced by *Pseudomonas protegens* CLP-6 have a broad-spectrum antagonistic effect against plant pathogens and were found to inhibit the growth of *D. chrysanthemi* by 80.9% (Zhao et al., 2023).

Competition for limited space and nutrients is a common biocontrol strategy. Given the low solubility of iron, numerous bacteria strains produce strong siderophores or iron chelators to enhance iron uptake. These complexes are specifically recognized by the receptors of the producer, limiting iron access to other species (Pandei et al., 2023). For instance, *Pseudomonas* Pf 2-79 produces iron-chelating siderophores, which limit the growth of SRP (Liao 2009). *Pseudomonas donghuensis* P482 is capable of inhibiting the

growth of SRP and attenuating disease symptoms. The antimicrobial efficacy is attributed to the synthesis of an iron-scavenging compound, 7-hydroxytropolone, as well as the release of antifungal volatile organic compounds and secondary metabolites (Krzyżanowska et al., 2023). The same biologically active compound has been identified in the CFS of *P. putida* PA14H7, which confirmed the bacteriostatic effect of 7-hydroxytropolone against *D. solani* (Munier-Lepinay et al. 2023).

An alternative antagonistic strategy involves disrupting the quorum-sensing mechanism of SRP. *Pectobacterium* sp. regulate their population density through a communication system reliant on signal molecules known as acyl-homoserine lactones (AHLs). This communication system plays a pivotal role in the expression of virulence factors, including the synthesis of cell-wall degrading enzymes. However, this system can be effectively disrupted by enzymatic degradation. In *Dickeya sp.* AHLs-QS does not regulate the production of cell-wall degrading enzymes, which are under the control of the Vfm-QS (Van Gijsegem at al., 2021). Some antagonistic bacteria are capable of interfering with quorum-sensing mechanisms. For example, *P. nitroreducens* W-7 degrades a wide range of AHLs, this effectively mitigates soft rot caused by *D. zeae* EC1, reducing tissue maceration in diverse host plants. Application of W-7's crude enzymes significantly decreases disease incidence and severity in host plants. Quorum quenching activity of *P. segetis* P6, by enzymatic degradation of QS molecules, has a broad spectrum of activity against *D. solani*, *P. carotovorum* and *P. atrosepticum* on potato and carrot (Rodriguez et al., 2020). Additionally, *Pseudomonas chlororaphis* L5 and *Enterobacter asburiae* significantly reduce the PCWDE production and virulence of *Dickeya*, by quenching VFM QS signal without affecting the growth of the plant (Liu et al., 2023).

Many of these isolates demonstrated their ability to inactivate SRP under laboratory settings. However, limited testing has been conducted under greenhouse or field conditions, and none of these strains have been formulated into a commercial product explicitly designed for combating SRP, notwithstanding ongoing testing initiatives (Czajkowski et al., 2011). An attempt to formulate a commercial product was made by Czajkowski et al. in 2020 with the formulation of an artificial consortium of five antagonistic strains against SRP (*Serratia plymuthica* strain A294, *Enterobacter amnigenus* strain A167, *Rahnella aquatilis* strain H145, *Serratia rubidaea* strain H440, *S. rubidaea* strain H469), named “The Great Five” (GF). The GF provides stable protection against SRP. Powder formulations, tested on potato tubers against soft rot

pathogens, showed a significant reduction in severity (62–75%) and incidence (48–61%) after 6 months at 8 °C (Krzyzanowska et al., 2019 and Maciag et al., 2020).

A promising alternative in the biocontrol of SRP is coming from phage therapy. Lytic bacteriophages against SRP can be isolated relatively easily from soils in which infected plants are grown. All currently described SRP bacteriophages belong to the order *Caudoviricetes* in one of three families: *Podoviridae*, *Siphoviridae* and *Myoviridae*, with the family *Myoviridae* being the most abundant (Zhu et al., 2022).

Bacteriophages such as LIMEstone and ΦD5 (Czajkowski et al., 2014 and 2016) have shown some potential for biocontrol of *D. solani*. In tissue culture and compost-grown potato plants, ΦD5 reduced infection by *D. solani* by more than 50% (Czajkowski et al., 2017). Phages LIMEstone1 and LIMEstone2 demonstrated a reduction in both disease incidence and severity in laboratory assays conducted on potato tubers. Moreover, in in-field experiments the application of the experimental phage treatment yielded higher crop output. (Adriaenssens et al., 2012). In a study conducted in Poland, nine bacteriophages infecting *D. solani* were isolated from soil samples. These bacteriophages completely halted the growth of *D. solani* in vitro and protected potato tuber tissue from maceration caused by the bacteria (Czajkowski et al., 2014).

The preliminary findings from these early successful studies should be approached with caution. Much of the work conducted served as proof-of-concept experiments using individual phage isolates, lacking extensive large-scale field trials. Furthermore, diverse phages were assessed in different laboratories, employing distinct experimental setups and operating under varying environmental conditions, making global data comparisons unfeasible. Consequently, it is premature to arrive at definitive conclusions regarding the potential utilization of lytic bacteriophages for controlling plant diseases caused by SRP (Czajkowski 2013).

1.5 Diseases caused by *Dickeya sp.*

D. solani causes black leg and top wilt of the growing potato plants and soft rot of the tubers. The most characteristic symptom of soft rot is the appearance of a water-soaked and translucent lesion that rapidly expands in both diameter and depth. The lesions usually develop in lenticels, at the site of stolon attachment, or in wounds. A brown or black pigment is usually visible at the margin of the decayed tissue, followed by the tuber maceration at a creamy consistency and a characteristic putrid odor caused by

decomposing bacteria. In optimal conditions, the rotting is completed in 20-72 hours. In storage, rotting can easily spread to adjacent tubers as the liquid cracking from the macerating tissue percolates among the lot (Czajkowski et al., 2011).

Blackleg is, generally, a soft rot that spreads from infected seed tubers into the stems of new potato plants, especially under wet conditions. The first symptom in the field is the reduction in plant emergence because infected tubers rot in the soil, and no plant appears. After emergence, the base of the shoots may become soft, brown to inky-black, and shriveled with dwarfed, stiff, and yellowish/bronzed leaves. Such plants often die prematurely, or their yield is reduced (Czajkowski et al., 2011; Reverchon and Nasser 2013).

1.6 *Dickeya* infection cycle

Reverchon and Nasser (2013) have carefully summarized the steps of infection of *D. dadantii* 3937, indicated as a model for *Dickeya sp.* (Glasner et al., 2011). The latently infected seed (mother) tuber is the major source of potato infection. In the spring, bacteria from these infected seeds disperse into the emerging young stems and roots. Once inside the plant, the bacteria penetrate intercellular spaces, breaking down the plant cell walls and causing maceration of the mother tuber. Following this, bacteria are released into the surrounding soil and are spread through soil water, contaminating adjacent progeny tubers (Czajkowski et al., 2010). The main steps of plant infection by *Dickeya* are: (i) adhesion to the plant surface; (ii) apoplast invasion; and (iii) plant cell wall degradation.

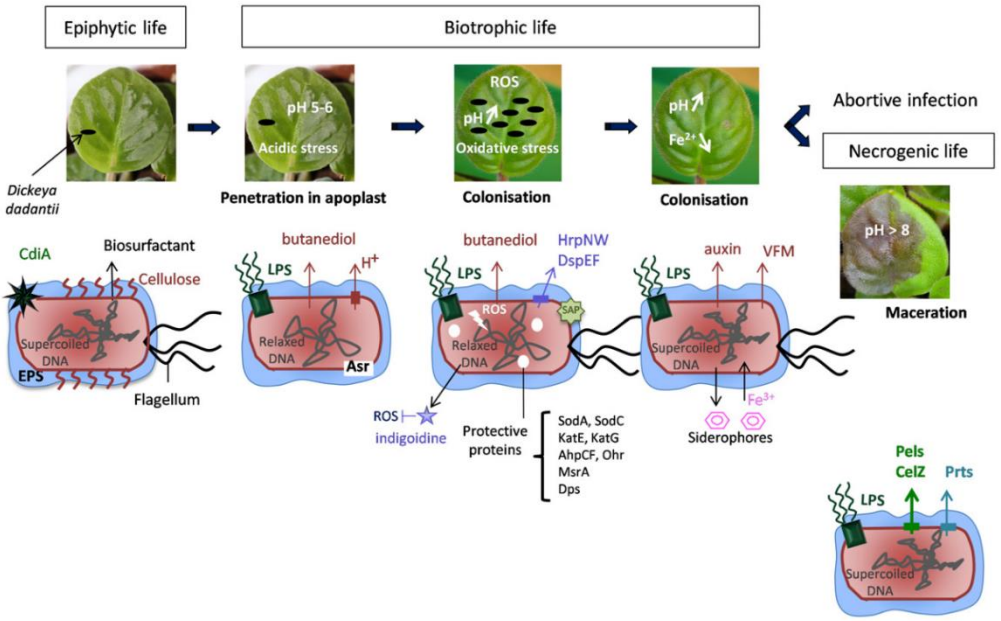

Figure 2 - A working model of Dickeya infection progress from asymptomatic to symptomatic stage (Reverchon and Nasser., 2013)

1.6.1 Adhesion to the plant surface

Dickeya sp. are able to reside as epiphytes on the plant surface and remain latent without causing any infection until they encounter favorable conditions for disease development, such as mild temperature, high humidity, and low oxygen levels (Lebeau et al., 2008).

Bacteria penetrate the plant host via natural openings (stomata, hydathodes, and emerging secondary roots), wounds, or contaminated tubers (Charkowski et al., 2012). At this stage, motility and chemotaxis play a crucial role in sensing the environment and moving toward the host. For instance, jasmonic acid, a key plant defense signal produced by the wounded tissue, acts as a strong chemoattractant and helps the bacterium to find its way in (Antunez-Lamas et al., 2009).

1.6.2 Apoplast invasion – asymptomatic phase

During the first few hours upon penetration, bacteria multiply in the intercellular space without causing any symptoms. This asymptomatic phase can last 8 hours, and it is required to adapt to the host environment and reach the population density sufficient to cause effective infection. The apoplast compartment is surrounded by the apoplastic fluid, which contains sufficient carbon metabolites and amino acids to sustain bacterial growth.

However, the apoplast is in an acidic environment (pH 4.0 – 6.5) and induces acidic stress response in *D. dadantii*. To cope with the acidic stress, bacteria (i) induce the *asr* gene for acid tolerance; (ii) use the respiratory chain and other antiport systems to pomp out protons; (iii) convert protons to hydrogen gas; (iv) reduce the production of flagellar motor to limit the proton re-entry; (v) remodel the cell wall by incorporating fatty acids and by modifying LPS. The LPS modification also confers resistance to antimicrobial peptides released by the plant defense system. (Reverchon and Nasser, 2013; Jiang et al., 2016).

The apoplast contains readily available sugars, mostly sucrose, glucose, and fructose. The presence of high assimilable sugars provokes the catabolite repression of other catabolic pathways and the induction of the butanediol pathway to avoid the accumulation of organic acids. This leads to a rise in pH and alkalization of the apoplast. The alkalization of the apoplast induces the secretion of siderophores (chrysobactin and achromobactin) to cope with the low availability of iron in the environment and improve iron uptake. Iron is essential in enzymatic reactions, and it is required for successful infection since mutants defective in siderophores production are unable to cause symptomatic infection (Franza and Expert., 2013). However, iron is also involved in the production of ROS. Therefore, iron homeostasis is very important during infection.

The production of ROS is the first line of plant defense. In response to the oxidative stress, most *Dickeya sp.* produce the bacterial pigment indigoidine that serves as ROS scavenger (Reverchon et al., 2016). Moreover, *D. dadantii* produces antioxidant enzymes such as superoxide dismutases, catalases, and alkylhydroperoxide reductase. For example, the activation of superoxide dismutase SodC has been observed in *D. solani* during the maceration of potato tubers (des Essarts et al., 2019).

Plants also produce a wide range of toxic antimicrobial compounds for *Dickeya sp.* Therefore, bacteria have evolved systems for the neutralization of such threats. For example, the SapABCDF transport system is responsible for the import and proteolytic degradation of antimicrobial peptides produced by the plants. (Lopez-Solanilla et al., 1998).

The transition from the asymptomatic to the symptomatic stage is marked by the production of cell wall-degrading enzymes (PCWDE). The timing for CWDE release is crucial; PCWDE to function requires a rise in pH, which is obtained by switching to

butanediol metabolism. However, butanediol is also perceived by the plant as a signal of infection and triggers plant systemic resistance. Therefore, the bacteria have to avoid a too-rapid and strong plant response while preparing for the infection. At this point, there are two possible scenarios: (i) the plant defense mechanisms are strong enough to stop the disease progression or (ii) the bacteria adapt to the adverse conditions and start to multiply until they reach a threshold population density and initiate the production of cell wall degrading enzyme leading to the establishment of the symptomatic phase.

1.6.3 Cell wall degradation – necrogenic life

A successful infection relies on the coordinated activation of numerous genes encoding virulence factors, including PCWDE which break down the plant cell wall and release nutrients used for bacterial growth. Among those, the production of pectinases marks the beginning of symptomatic infection. These enzymes cause maceration and rotting of parenchymatous tissues on all plant organs, resulting in cell lysis and necrosis.

In plant tissue, *Dickeya sp.* encounter low oxygen availability, which is a key factor for the induction of virulence genes (Hugovieux-Cotte-Pattat et al., 1992). Transcriptional profiling revealed that ca. 10% of *D. dadantii* 3937 genes are differentially expressed under oxygen-limited conditions in comparison with normal oxygen concentration (Babujee et al., 2012). Anaerobiosis is perceived by bacteria through the FNR, NarXL and ArcAB systems. FNR is a transcriptional regulator containing an iron-sulfur cluster, which forms dimers in the absence of oxygen. NarXL is responsible for activating genes related to nitrate/nitrite catabolism under anaerobic conditions while simultaneously repressing genes associated with other anaerobic respiratory and fermentative pathways. The ArcAB system monitors the oxidation state of ubiquinones in the aerobic respiratory chain and, under anaerobic conditions, inhibits genes necessary for aerobic metabolism. Notably, this system is non-functional in *D. dadantii*, making it an exception among *Dickeya sp.* (Babujee et al., 2012).

While pectinases are necessary for effective infection, they are not solely responsible for it. Analysis of the *D. solani* genome has revealed several additional genes that may play roles in pathogenicity and toxin production. These genes include clusters encoding polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPS), amino acid adenylation domains, and proteins transported through T5SS/T6SS systems (Garlant et al., 2013; Pédron et al., 2014).

In the apoplast, bacterial growth was sustained by available simple sugars produced by the plant. The success of the infection process depends on the capacity to swiftly and effectively transition from glucose to pectin catabolism (Leonard et al., 2017). Therefore, PCWDEs released by *Dickeya* serve a dual purpose, acting as virulence factors and nutrient sources. This dual functionality highlights that the regulation of gene expression is influenced by both metabolic and virulence regulators.

1.7 Plant cell wall degrading enzymes - PCWDE

The plant cell wall is composed of cellulose/hemicellulose embedded in a matrix of acidic polysaccharides, commonly called "pectin." Pectin represents 30-50% of the cell wall, marking its importance for tissue stability. For this reason, pectin is also the target of many phytobacteria. Pectin has a complex structure made up of three polysaccharides: PGA (polygalacturonic acid), RGI (branched rhamnogalacturonan I), and RGII (rhamnogalacturonan II). PGA, RGI and RGII are highly branched polymers, with variable side chains, methylations and esterifications (Hugouvieux-Cotte-Pattat., 2016).

Dickeya sp. secrete a wide range of PCWDE, which are responsible for the infection symptoms (maceration). PCWDEs include pectinases and additional enzymes such as cellulases, xylanases and proteases. While participating in the infection, these additional enzymes are not essential for pathogenesis, which is to be attributed to the activity of pectinases.

The pectinases produced by *Dickeya sp.* were described by Hugovieux-Cotte-Pattat and colleagues in 1996 and reviewed in 2014. In general, pectinases are enzymes able to cleave the glycosidic link or the methyl-ester bonds of pectin. *Dickeya sp.* produce a wide range of pectinases targeting different constituents of pectin and with diverse modes of action: pectate lyase (Pel), pectin lyase (Pnl), pectin methyl esterase (Pme), polygalacturonase (Peh) and pectin acetyl esterase (Pae).

Pectate lyases are the main pectinases involved in the pathogenesis. Similar to other exoenzymes, the abundance of Pels varies across different species, subspecies, and strains. Typically, pectate lyases exhibit optimal activity at basic pH levels ranging from 7.3 to 9.5 and have a strict dependence on a divalent cation, with calcium ions (Ca^{2+}) being the preferred choice in most cases.

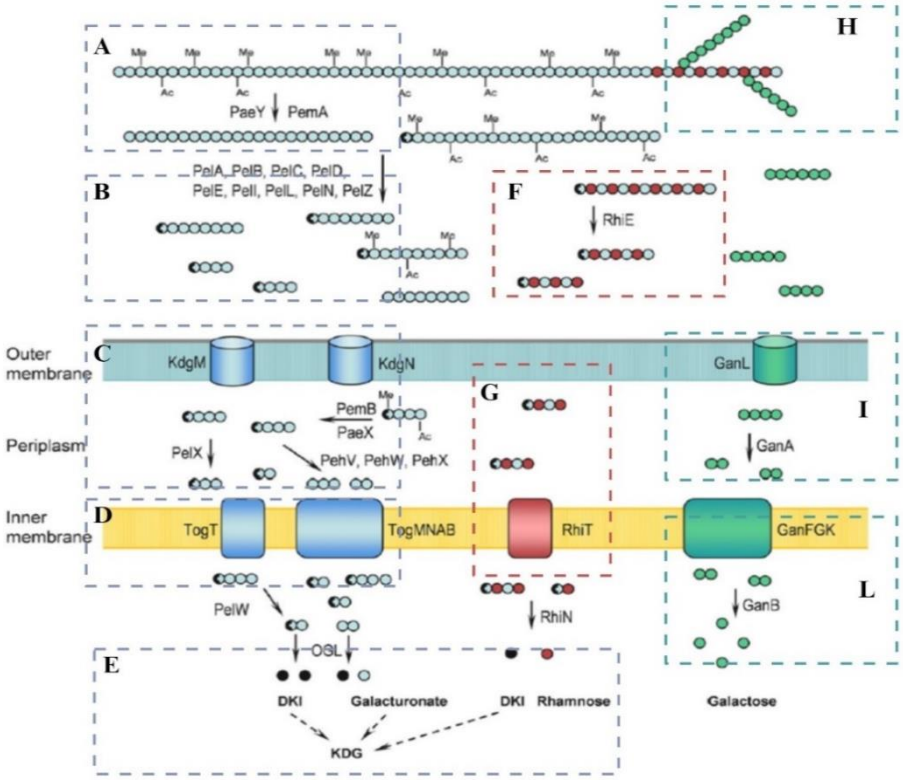

Figure 3 - Schematic pectin degradation pathway in D. dadantii (Hugouvieux-Cotte-Pattat et al., 2014) A - Removal of Methyl-esterification and Acetylation; B - Cleavage of Polygalacturonate (PG) Backbone; C - Periplasmic Processing; D - Cytoplasmic Uptake; E - Cytoplasmic Metabolism; F - Cleavage of Rhamnogalacturonan I (RGI) Backbone; G - Periplasmic Processing and Cytoplasmic Uptake of RGI; H - Metabolism of Galactan Side Chains; I - Periplasmic Processing and Cytoplasmic Uptake of Oligogalactans; L – Cytoplasmatic Metabolism

In plant tissue, *Dickeya sp.* encounter low oxygen availability, which is a key factor for the induction of virulence genes (Hugovieux-Cotte-Pattatet al., 1992). Anaerobiosis is perceived by bacteria through the FNR, NarXL and ArcAB systems (Babujee et al., 2012)

Based on genomic analysis, *D. solani* produces nine pectate lyases (PelA-E, PelI, PelL, PelZ). PelADE can cleave PGA main chains, and PelBC are able to cleave esterified polymers. The bacteria also produce a pectin acetyl esterase (PaeY) and two pectin methyl-esterases, the secreted PemA and the membrane protein PemB, which are able to cleave methyl and acetyl groups, leaving a suitable substrate for the action of pectate lyases. This species also produces exo-polygalacturonase (PehVWX) and endo-polygalacturonase (PehN) (Golanowska et al., 2018; Hugovieux-Cotte-Pattat 2014).

The enzymes are exported via a type II secretion system (T2SS) named “Out,” large transmembrane proteins associated with both the inner and outer membranes. The Out system not only facilitates the secretion of endo-pectate lyases and esterases but also plays

a role in the transportation of the rhamnogalacturonate lyase RhiE and the cellulase CelZ (Hugovieux-Cotte-Pattat., 2016)

The pectinases cleave the glycosidic bond between two D-galacturonate residues of the PGA, releasing the unsaturated oligogalacturonates then traverse the periplasm through the porins KdgM and KdgN. In the periplasmic space, PemB and PaeX remove the remaining methyl and acetyl groups, respectively. Subsequently, the exo-pectate lyase PelX and exo-polygalacturonases PehV, PehW, and PehX further cleave oligomers. These smaller oligomers enter the cytoplasm through transporters TogT and TogMNAB. Within the cytoplasm, enzymes PelW and OGL collaboratively generate two monomers, 5-keto-4deoxyuronate (DKI) and galacturonate, which undergo further metabolism into 2-keto-3deoxygluconate (KDG).

Simultaneously, the rhamnogalacturonan I (RGI) is cleaved by the rhamnogalacturonate lyase RhiE. These oligomers utilize the transporter RhiT to access the cytoplasm, where the enzyme RhiN cleaves the terminal unsaturated galacturonate residue.

Additionally, the galactan side chains of RGI are metabolized, with oligogalactans entering the periplasm through the GanL porin. The periplasmic endo-galactanase GanA produces short oligomers, utilizing the GanFGK transport system to traverse the inner membrane. Ultimately, the cytoplasmic exo-galactanase GanB cleaves oligogalactans into individual galactose units (Hugovieux-Cotte-Pattat., 2016).

1.8 Importance of secretion system in the pathogenesis of *Dickeya sp.*

The rapid induction of genes encoding pathogenicity factors within bacterial cells is only impactful if these factors can be efficiently transported to the extracellular environment. To achieve this, SRP possess all six secretion systems characteristic of Gram-negative bacteria (Glasner et al., 2011; Garlant et al., 2013; Pedron et al., 2014).

The Type I Secretion System (T1SS) is responsible for transporting proteases directly from the cytoplasm to the extracellular space in a single step. While extensively studied in *Dickeya sp.*, it appears to play a relatively minor role in their pathogenicity (Golanowska and Lojkowska, 2016).

The Type II Secretion System (T2SS) is also known as the "Out" system and is essential for the pathogenesis of *Dickeya sp.* as it allows the secretion of CWDE (Py et al., 1991). Secretion is a two-step mechanism: the first step is a Sec-dependent protein export that allows the release of unfolded proteins in the periplasm. Then, the proteins are folded by chaperones and transported through the T2SS in the extracellular space.

In phytopathogenic bacteria, the Type III secretion system (T3SS), also known as the hypersensitive response and pathogenicity (Hrp) system, serves as a critical virulence factor by facilitating the secretion and translocation of effector proteins into host cells. Genome sequencing of *D. dadantii* 3937 has confirmed the presence of a complete set of genes encoding the T3SS apparatus, which has been implicated in the pathogenicity of this bacterium (Yang et al., 2008).

In contrast to other secretion systems, the Type IV secretion system (T4SS) stands out for its capacity to transport nucleic acids alongside proteins into plant and animal cells, as well as into yeast and other bacteria (Christie et al., 2005).

The functions of proteins secreted by the Type V Secretion System (T5SS) and Type VI Secretion System (T6SS) are diverse; they include serine proteases, lipases, cytotoxins, invasins, and adhesins. Collectively, these proteins contribute to various aspects of bacterial fitness, including aggregation, biofilm formation, and virulence (Grijpstra et al., 2013). Additionally, T5SS have been associated with a phenomenon known as contact-dependent growth inhibition, wherein toxic proteins are directed toward neighbouring bacteria upon physical contact (Ruhe et al., 2013). *D. solani* encodes a distinctive arsenal of T5SS and T6SS related toxin-antitoxin systems (Pedron et al., 2014).

1.9 *Dickeya* virulence regulatory network

Expression of virulence factors in *Dickeya sp.* is under the control of an intricate regulatory pathway that acts at transcriptional and post-transcriptional levels. This tight regulation allows bacteria to optimize the use of energy in response to changes in environmental conditions (including temperature, pH, oxidative and osmolarity stresses), growth phase (transition from exponential to stationary phase), population density, and the metabolic status of the cell (transition from sugar-based metabolism to pectin catabolism). A second level of complexity in the regulation of *Dickeya sp.* virulence is created by the DNA topology. It has been shown that DNA topology changes during the infection from supercoiled to coiled structure, orchestrated by the NAPs. PecT has been

found to bind preferentially to relaxed pel DNA promoters (Herault et al., 2014). For instance, early virulence factors involved in plant surface colonization, such as cellulose biosynthesis and the type V secretion system, are preferentially produced when DNA is supercoiled. In contrast, genes involved in resistance to environmental stresses, such as acidic and oxidative stress, are activated upon DNA relaxation (Reverchon and Nasser, 2013).

The main regulators of virulence factors in *Dickeya* include nucleoid-associated proteins (NAPs); transcriptional factors (Kdgr, PecS, PecT); cAMP receptor protein (CRP); two-component systems (GacA/GacS); and quorum sensing (VFM and Exp/ExI).

1.9.1 NAPs – nucleoid-associated proteins

NAPs are the most abundant regulators that coordinate the architecture and transcription of DNA in bacteria (Amemiya et al., 2021).

NAP Fis has been identified as a key regulator of virulence in several pathogenic bacteria, including *D. dadantii*. It plays a crucial role in the transition from asymptomatic to symptomatic phase activating the early virulence genes and repressing *pel* genes. This control occurs through a growth-dependent mechanism where Fis binds directly to the promoter region of the interested genes. During the early exponential phase, Fis is synthesized in large quantities, binds to the promoter of the virulence genes, and hinders CRP and RNA polymerase binding sites. At the same time, it promotes flagella motility and the defense mechanisms against the plant antimicrobial compounds. At the entering of the stationary phase, Fis becomes almost undetectable leading to de-repression of the pel genes (Duprey et al., 2014).

NAP H-NS is a global regulator of gene expression in response to environmental conditions including temperature, pH, oxidative and osmolarity stress (Dillon and Dorman., 2010). H-NS has been shown to activate the synthesis of *pels* genes by negatively affecting the synthesis of PecT repressor and activating the production of the CRP activator (Reverchon and Nasser., 2013).

1.9.2 Transcriptional factors

KdgR

KdgR is the transcriptional repressor of 32 target operons involved in pectin degradation. In the absence of pectin, CWDE genes are subjected to only basal transcription. This transcriptional leakage allows the initiation of the induction cycle. In fact, Kdgr acts as a pectin sensor. When bacteria encounter pectin, the intracellular metabolites of PGA degradation provoke the dissociation of KdgR from its binding sites, activating the transcription of genes involved in the pectin catabolism. The KdgR repression works in synergy with NAP-Fis repression and CRP activation protein (Hugovieux-Cotte-Pattat 2016., Reverchon and Nasser 2013).

PecS

PecS is a transcription factor of the MarR family which regulates over 13% of the pathogen genome. The PecS regulon involves plant cell wall-degrading enzymes, multiple secretion systems and flagellar components, and indigoidine biosynthesis (Hommais et al., 2008). PecS directly prevents the early expression of numerous virulence factors and responds to the plant immune system signals by releasing its repression in the presence of these signals (Reverchon et al., 2016)

PecT

PecT is a transcriptional factor of the LysR family. PecT modulates *pel* gene expression in a temperature-dependent manner; the binding of PecT to *pel* promoters and repression increases with increasing temperature. The activity of PecT has been linked to DNA topology, as it exhibits a higher affinity for relaxed DNA. At elevated temperatures, changes in DNA supercoiling enhance PecT binding affinity to pel regulatory regions, thereby suppressing pel gene expression. (Reverchon et al., 2016) In *D. solani*, PecT is considered to play a crucial role in allowing adaptation to a wider range of temperatures, which has been linked to the rapid spread of this pathogen in Europe (Potrykus et al., 2014).

1.9.3 cAMP Receptor Protein (CRP)

Switching from the asymptomatic to symptomatic phase also marks the transition from a metabolism based on the sugars present in the apoplast to the catabolism of pectin. This metabolic change is tightly regulated by the interaction between the KdgR pectin sensor-

repressor and the CRP activator. The major role of CRP is to favor the utilization of the most efficient metabolic carbon source. CRP is activated by binding the cAMP. In the presence of glucose, the low intracellular level of cAMP prevents the activation of CRP in favor of the consumption of glucose. During the symptomatic phase the levels of glucose decrease, leading to an increase of cAMP. The active complex cAMP-CRP activates the expression of *pel* genes. Therefore, CRP plays a crucial role in coordinating the virulence of *Dickeya* according to nutrient conditions.

1.9.4 Two-component systems

The GacA/GacS two-component system is essential for *Dickeya dadantii* virulence, as mutants are impaired in the production of pectin lyases and cellulases (Lebeau et al., 2008). The regulation of GacA is associated with carbon catabolism and the regulatory network involves CRP and the non-coding RNA Rsm. CRP represses GacA which activates RsmB (Song et al., 2023). RsmA and RsmB are small noncoding RNA implicated in the post-transcriptional regulation of CWDE and T3SS (Yang et al., 2008).

A second level of complexity in the regulation of *Dickeya sp.* virulence is created by the DNA topology. It has been shown that DNA topology changes during the infection from supercoiled to coiled structure, orchestrated by the NAPs. PecT has been found to bind preferentially to relaxed pel DNA promoters (Herault et al., 2014). For instance, early virulence factors involved in plant surface colonization, such as cellulose biosynthesis and the type V secretion system, are preferentially produced when DNA is supercoiled. In contrast, genes involved in resistance to environmental stresses, such as acidic and oxidative stress, are activated upon DNA relaxation (Reverchon and Nasser, 2013).

1.9.5 Quorum sensing

The achievement of a successful infection also depends on the population density. At a low population density, the release of virulence factors can trigger the plant's immune defense too early, leading to the abortion of infection. Therefore, as Gram-negative bacteria, *Dickeya sp.* employ a mechanism known as quorum sensing (QS) to coordinate the expression of virulence-related genes based on population density, ensuring timely and coordinated infection.

Gram-negative plant-pathogenic bacteria employ a quorum-sensing mechanism that relies on the synthesis and detection of N-acyl-homoserine lactones (AHLs) as signaling

molecules. The AHL-mediated QS system is widely observed and extensively studied among these bacteria, including various *Dickeya* species, with the exception of *D. paradisiaca* (Potrykus et al., 2014). QS generally controls the expression of genes involved in virulence and social behaviour, such as toxin production, biofilm formation, motility, and conjugation. However, in pathogenic bacteria, QS also plays a major role in the regulation of virulence factors (Papenfort et al., 2016; Baltenneck et al., 2021).

The AHLs are produced by the ExpI synthases, which belong to the LuxI family of proteins. At low population density, basal transcription of ExpI allows the secretion of AHLs, which accumulate in the medium. When the external concentration increases above a certain threshold, AHLs freely diffuse through the membranes and act by directly binding to the transcriptional factor ExpR, which belongs to the LuxR family. ExpR acts as a repressor and blocks the transcription of the regulated genes in the absence of AHLs. ExpR also functions as an autoinducer, amplifying the signal. *Dickeya dadantii*, *D. solani* and *D. dianthicola* secrete two types of AHLs N-3-oxohexanoyl-homoserine lactone (3OC6-HSL), hexanoyl-homoserine lactone (C6-HSL) (Crepin et al., 2012).

AHLs control the expression of more than 70 regulators, positively or negatively, which are involved in motility, secretion of virulence factors, and plant colonization (Baltennek et al., 2021). However, the involvement of AHLs-QS in *Dickeya sp.* virulence is an object of discussion, as the degree of control of virulence varies among different species. For instance, in *D. dadantii*, it has been observed that the QS ExpR/ExpI system does not play a role in the production of PCWDEs and does not significantly influence its ability to cause maceration (Castang et al., 2006). Moreover, some *Dickeya* species, such as *D. paradisiaca*, do not encode the AHL synthase *expI*. On the other hand, AHLs-QS plays a crucial role in protease production in *D. solani*, where the *expR* deletion mutant showed reduced virulence in vitro and in planta and impaired swimming and swarming motility. However, it must be noted that the degree of control of virulence in *D. solani*, varies among the strains, the host plant and the virulence assays, providing contrast results (Potrykus et al., 2014).

Investigations on the ambiguous role of AHLs-QS in the virulence of *Dickeya sp.* have led to the discovery of an additional QS that is present in all *Dickeya sp.* except for *D. paradisiaca*. The VFM (virulence factor modulating) system is a second QS system encoded in a cluster of 25kb, situated between the *expR* gene and genes encoding for

indigoidine. The cluster contains 26 genes involved in the production, sensing, and transduction of the QS signal. In *D. dadantii*, the VFM-QS regulates the production of PCWDEs and virulence (Nasser et al., 2013b). Despite several attempts of isolation and characterization, the nature of the VFM signal molecule has not been elucidated yet. However, a few characteristics are known: (i) the signal is an extracellular peptide as it can be sensed in the CFS; VFM is a non-ribosomally synthesized peptide; different strains produce and respond to different types of signaling molecules; the signal is produced from the early to the exponential phase of growth.

The VFM signal accumulates in the medium until a certain threshold is reached, then it is received by the two-component system VfmH-VfmI, which activates a signaling cascade orchestrated by the activation of *vfmE* that activates the transcription of genes involved in virulence and autoregulates, in a positive feedback loop, the transcription of the *vfm* operons (Nasser et al., 2013a).

The VFM system is regulated by PecS, as PecS functions as a repressor to avoid activation at the early stages of infection (Pedron et al., 2018). Moreover, Fis has been found to be able to bind and activate the vfm operon directly.

In bacteria, regulation of social behaviour, including motility and biofilm formation, is intricately associated with levels of c-di-GMP. When intracellular c-di-GMP levels are low, bacteria tend to adopt a planktonic lifestyle, whereas higher levels promote a sessile existence, such as biofilm formation. Recent studies suggest that VfmE works as a c-di-GMP effector, integrating population density and the c-di-GMP levels in the regulation of virulence factors. Indeed, VfmE has a binding site for c-di-GMP, resulting in VfmE repression of Pels genes in the presence of high levels of c-di-GMP. (Banerjee et al., 2022).

2 Plant Growth Promoting Rhizobacteria

The plant rhizosphere, the thin layer of soil surrounding the roots, serves as a rich hot spot for bacteria which are abundantly present due to the high availability of nutrients in the proximity of the plant roots. These bacteria play an active role in interacting with plants and other microbial communities. They provide a multitude of benefits to the plant, including enhancing nutrient availability, alleviating abiotic stress, fostering plant growth, stimulating plant defense mechanisms, and actively fighting plant pathogens. Consequently, these bacteria have been termed Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper and Schroth 1981). To date, the definition of PGRPR has evolved to include bacterial strains capable of meeting at least two out of three criteria: (i) robust colonization, (ii) stimulation of plant growth, and (iii) biocontrol capabilities (Bhattacharyya and Jha., 2012)

In recent years, PGPR found several applications in agriculture as they appear as a valuable alternative to the use of chemicals and pesticides in crop management. Application of PGPR is viewed as able to slow down the emergence of resistant pathogens and generally considered safe and environmental friendly (Blake et al., 2021).

Numerous bacterial species documented in the literature serve as PGPR, demonstrating effectiveness in enhancing plant growth. However, in recent years representatives of *Pseudomonas* and *Bacillus* have been attracting the main attention. Despite significant advances in research, the commercial use of *Pseudomonas* remains constrained by challenges in formulating stable preparations. Consequently, endospore-forming bacteria of the *Bacillus* genus remain a more viable alternative.

At present, *Bacillus* species are by far the most widely used bacteria in bioformulation in America. The initial market application dates back to 1897 with a product named Alinit, produced by what is now Bayer AG, containing spores of *Bacillus subtilis*. The beneficial application of *Bacillus subtilis* are also exploited in currently available products, including Serenade (Agraquest Inc., Davis, CA, US), Subtilex (Becker Underwood, Ames, IA, US) and Kodiak® (Gustafsson, Inc., Plano, TX, US) (Borris, 2011).

In Europe, the successful application PGPR is limited by regulatory constraints. Biopesticides follow the same registration procedure of chemical pesticides (EC No 1107/2009), therefore, the costly risk assessment and long-term evaluation keeps these products out of the market. For instance, the inclusion of the microorganism

Pseudomonas chlororaphis (the active ingredient in Cedomon®) in EU took over 8 years and required an investment exceeding EUR 2.5M. Furthermore, the evaluation of dossiers in the EU takes more than 70 months, compared to approximately 23 months for similar products in the US (Borris, 2011).

The EU Green Deal, which is currently under review, aims to offer alternative tools to replace chemical plant protection products with more sustainable options. Therefore, we can expect that new regulatory requirements for microorganisms used as biocontrol agents will facilitate faster access to the EU market.

2.1 The *Bacillus* genus

The genus *Bacillus* was first described and classified by Ferdinand Cohn in 1872 as part of the Family *Bacillaceae*, which belongs to the *Bacillota* phylum (previously named *Firmicutes*). These bacteria are characterized by their rod-shaped morphology, positive catalase reaction, ability to form endospores and aerobic or facultative anaerobic metabolism. The genus *Bacillus* currently includes 293 named species/subspecies (Patel and Gupta, 2020), majority of them are harmless saprophytes. However, it also includes notable human pathogens such as *Bacillus anthracis*, the causative agent of anthrax, and *B. cereus*, which is associated with food poisoning.

Although *Bacillus* sp. typically inhabit soil, they demonstrate remarkable adaptability to colonize nearly any environment. They can be found in water, air, various surfaces, plant rhizospheres, and the gastrointestinal tract of animals, as well as in numerous extreme environments (Nicholson et al., 2000).

Their capability to survive in harsh conditions is attributed to their ability to transition from vegetative growth to sporulation (spore-formation). Sporulation is a complex and energy-consuming process that serves as a last chance of survival for bacteria facing adverse environmental conditions. Endospores (from now referred as spores) are engulfed in a multilayer shell, the coat, which sequesters the bacterial genome in a safe space. The coat, which is made of more than 70 different proteins, allows spores to resist to environmental stress including (but not limited to): dry and wet heat, desiccation (including vacuum), UV and gamma radiation and chemical disinfection. Bacteria can survive as dormant spores for years until conditions become favourable again for vegetative growth (Setlow., 2014).

From a biotechnological perspective, *Bacillus* bacteria are widely utilized in industry due to their Generally Recognized as Safe (GRAS) status (Food and Drug Administration, www.fda.gov). Their industrial application depends on their ability to produce a diverse array of secondary metabolites, including enzymes, amino acids, vitamins, surfactants, and bioactive compounds. For instance, *B. licheniformis* stands out for its large-scale production of extracellular enzymes including β-lactamase, thermostable α-amylase, and protease (de Boer et al., 1994). Moreover, *B. licheniformis* produces a wide range of antimicrobial compounds with medical applications, showing promise for being used as probiotics in the treatment of dysbacteriosis (Shleeva et al., 2023).

In the food industry, *Bacillus amyloliquefaciens* is highly valued for its production of hydrolytic enzymes, such as β-glucanases and metalloproteases, used in wine and brewing, as well as food processing. Additionally, other *Bacillus* species, such as *B. polymyxa*, and *B. coagulans*, produce various debranching enzymes used to manufacture food sweeteners. *Bacillus* species also produce bioactive compounds like antimicrobial peptides (AMPs) and bacteriocins, such as tyrocidine and bacitracin, which have applications as food preservatives and pharmaceuticals. Moreover, *Bacillus* spores have been widely used as probiotics, the best example being *B. clausii*. Spores of *Bacillus* allow easy storage at room temperature making it a better choice compared to *LactoBacillus* (Harirchi et al., 2022).

In agriculture, the resistance and longevity of spores make *Bacillus* sp. a preferred candidate as MCA. *Bacillus* sp. isolated from the rhizosphere act as promising PGPR due to their ability to enhance plant growth, stimulate systemic resistance in host plants, and generate a wide spectrum of antimicrobial compounds, including antibiotics, lipopeptides, and enzymes.

2.2 *Bacillus subtilis* – an ally in the fight against phytopathogens

Bacillus subtilis is a Gram-positive, non-pathogenic, spore-forming, motile, and capable of anaerobic growth bacterium. The group consists of rod-shaped small cells, typically 2–6 µm long and less than 1 µm in diameter (Errington and van der Aart., 2020). *B. subtilis* is the model organism of Gram-positive bacteria, and *B. subtilis* 168 is considered the type strain of the group. *B. subtilis* 168 is also one of the first bacteria genomes to be fully sequenced (Kunst et al., 1997) and it is still one of the best-annotated

genomes. The pangenome encounters about 6250 genes and the core genome about 2500 genes (Borris et al., 2018).

Like other members of the *Bacillus* genus, *B. subtilis* is capable of forming resilient spores that are highly resistant to biotic and abiotic stress. Coupled with its ability to produce antimicrobial compounds and interact with plant roots, this makes it a promising candidate for agricultural applications. In fact, *B. subtilis* is one of the most widely used and studied PGPR.

Several studies have demonstrated that *B. subtilis* environmental isolates are capable of conferring biotic and abiotic stress tolerance to plants, enhancing plant growth and soil health. *B. subtilis* can directly support plant growth by (i) providing easy access to nutrients, (ii) producing plant growth hormones, and (iii) enhancing plant resistance to stress conditions.

2.2.1 Improving nutrient availability

Nutrient availability is crucial for plant growth and seed germination. However, many essential nutrients and trace elements, such as iron, phosphorus, and nitrogen, often exist in the soil in forms that are inaccessible to plants. Therefore, plants depend on PGPR to convert these compounds into bioactive forms. For example, *B. subtilis* has been found to fix atmospheric nitrogen and promote the colonization of symbiotic rhizobacteria in plant roots (Ben Khedher et al., 2021). Additionally, it aids in phosphorus uptake by producing phosphatases and organic acids that acidify the soil environment, facilitating the conversion of inorganic phosphate into a form that plants can readily absorb (Saeid et al., 2008). Moreover, *B. subtilis* produces siderophores, which sequester iron from competing bacteria, thereby enhancing iron mobility and absorption by plants (Zhang et al., 2009).

2.2.2 Regulation of plant hormone homeostasis

B. subtilis is also capable of inducing cell division and plant growth by directly influencing plant hormone homeostasis. For example, *B. subtilis* 26D produces cytokines that have been found to be directly responsible for inducing plant growth (Sorokan et al., 2021). *B. subtilis* GB03 and SYST2 release organic volatile compounds (VOCs) that have been demonstrated to activate the genes involved in the auxin synthesis in *A. thaliana* and

tomato plants, respectively. Moreover, VOCs produced by SYST2 strain activate the synthesis of cytokines in tomato plants (Zhang et al., 2007; Tahir et al., 2017).

2.2.3 Tolerance to abiotic stress

In addition to directly promoting plant growth, *B. subtilis* is able to support plant growth by increasing plant tolerance to abiotic stress. In particular, strains GOT9, HAS31, and 10-4 have been shown to enhance tolerance to drought in different plants, including potato and wheat. The resistance is achieved through the modulation of the plant stress-response genes (Lastochkina et al. 2020; Han et al., 2014; Lastochkina et al., 2014).

2.2.4 Antibiosis

From the biotechnological point of view the interest in *B. subtilis* relies on its ability to produce secondary metabolites and to protect plants from pathogen infections through direct antibiosis and indirectly by induced plant systemic resistance (ISR).

Soil represents a very competitive environment, therefore, the ability to produce secondary metabolites provide bacteria a competitive advantage. It has been estimated that approximately 4-5% of the genome of *B. subtilis* contain genes responsible for producing and secreting antimicrobial compounds (Stein, 2005). Soil isolates of *B. subtilis* are able to produce a wide range of lipopeptides, exoenzymes and VOCs. While these compounds are not essential for growth they act as biological weapons.

Among these antimicrobial compounds, LPs (cyclic lipopetides) are of major interest for potential applications in biotechnology and biocontrol. LPs families include: surfactin, iturin and fengycin. LPs are non-ribosomal synthesized peptides (NRPs) which are produced through a sequential addition of amino acids residue. These highly flexible biosynthetic pathway supports the high heterogeneity of the produced molecules.

The most studied active molecule produced by *B. subtilis* is surfactin. Surfactin was first identified in 1968 (Arima et al., 1968), and it is known as one of the most potent biosurfactants. Surfactin is a cyclic peptide chain with seven amino acids with a β-hydroxy fatty acid chain of thirteen to fifteen carbons. Surfactin works by reducing the surface tension between two phases, enhancing motility and biofilm formation. However, surfactin is well known, especially for its antimicrobial (Bais et al., 2004) and antiviral (Vollenbroich et al., 1997) properties. The antimicrobial properties of surfactin have been recently reviewed by Chen and colleagues in 2022. Briefly, surfactin is an amphipathic

molecule that can integrate into the lipid layers; then, it acts as a detergent, provoking the dehydration of the polar head groups of phospholipids. These changes lead to membrane instability and disruption. For instance, surfactin, derived from *B. subtilis* R14 strain (Fernandes et al., 2007), exhibits antagonistic effects against multidrug-resistant bacterial strains, including *Alcaligenes faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *methicillin-resistant Staphylococcus aureus*. Surfactin finds applications in the biocontrol of several plant pathogens including *Pseudomnas Syring*ae and *Acidovorax citrulli*. In addition to direct antibiosis, surfactin indirectly protects the plant from pathogen infection by promoting the swarming and biofilm colonization of protecting *B. subtilis* on plant roots, while reducing the adherence of pathogenic cells (Fan et al., 2017)

Among LPs, the Iturin family comprises cyclic lipopeptides consisting of seven amino acids linked to a β-amino fatty acid chain containing fourteen to seventeen carbons (Ongena and Jacques, 2008). Their amphiphilic nature strongly indicates interaction with cell membranes. However, unlike surfactin, iturin exhibits only limited antibacterial activity but demonstrates a broader spectrum of antifungal effects. Furthermore, its mechanism of action differs; rather than directly disrupting cell membranes, iturin induces osmotic perturbation by forming ion-conducting pores. For example, iturin A produced by *B. subtilis* RB14 has a direct inhibitory effect on *Rhizoctonia solani*, the causal agent of dumping-off. Iturin produced by *B. subtilis* WL-2 has also a strong inhibitory activity on *P. infestans* mycelium growth (Wang et al., 2020).

Fengycin is an amphiphilic cyclic peptides made of seven amino acids linked to a β-hydorxy fatty acid od fourteen to eighteen carbons. has an antifungal activity against filamentous fungi. This activity has been demonstrated in strain GA1, which is able to protect damaged apple fruits against the diseases caused by *Botrytis cinerea* and in strain 9407 which shows antifungal proprieties against *Botryosphaeria dothidea*, the causal agent of apple ring rot (Tourè et al., 2003; Fan et al., 2017). Moreover, fengycin, together with iturin, isoleted from stain ATCC 6633, has been shown to suppress infection caused by *Podosphera fusca* on melon leaves (Romero et al., 2007).

Surfactin and fencycin also act as elicitors activating ISR in plants, making the host more resistant to possible pathological infections. The triggering of ISR is linked with cell wall degradation, the synthesis of new proteins including glucanases and chitinases, and the production of phytoalexins associated with disease resistance. For instance, a significant

protective effect has been obtained by treating bean ant tomato leaves with surfactin and fencycin produced by *B. subtilis* S499 (Ongena et al., 2007).

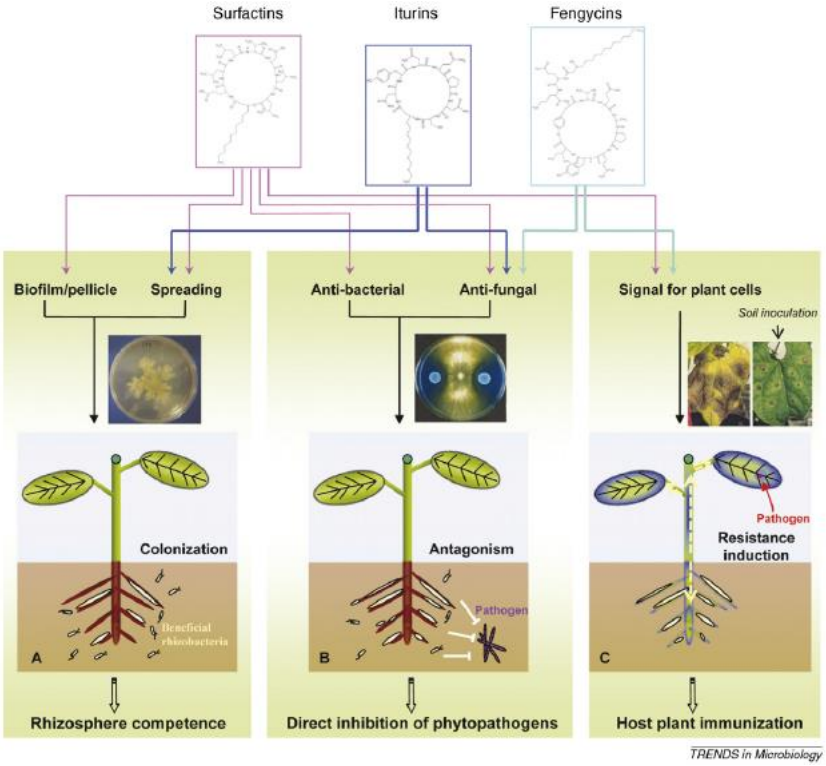

Figure 4-Overview of Bacillus subtilis LPs interactions in the biocontrol of plants diseases. Mechanisms of biological control are summarized as follows (A) plant root colonization, spreading and biofilm formation; (B) direct antagonism; (C) activation of ISR. (Ongena et al., 2007).

2.3 *Bacillus subtilis* arsenal: antimicrobial compounds secreted by *B. subtilis*

Despite having major applications in agriculture, LPs represent only a small fraction of the antimicrobial compounds' arsenal secreted by *B. subtilis*. In addition to LPs, *B. subtilis* sp. secrete other bioactive molecules, including ribosomal peptides (RPs), polyketides (PKs) and volatiles compounds (VOCs).

RPs are obtained by short precursors that undergo post-transcriptional modification. This synthesis mechanism explains the wide diversity of chemical structures and activity of this group. Bacteriocins are among the most extensively studied RPs. They are commonly secreted by nearly all bacteria; in fact, it is estimated that approximately 99% of bacteria and archaea produce at least one type of bacteriocin. Lantibiotics are a class of bacteriocins characterized by an inter-residual thioester bond that confers the lantibiotic structure. Subtilin is the most studied lantibiotic as it is structurally related to the most

known nicin produced by *L. lactis*, used as food preservative and approved by FDA(Ross et al., 2002). Bacteriocin are generally effective against Gram-positive bacteria, and their mode of action relies on pore formation, which disrupts the membrane potentials, causing cell lysis (Oscariz et al., 2000).

B. subtilis sp. produce a wide range of enzymes with antimicrobial activity: lytic enzymes and enzymes involved in quorum quenching. The lytic enzymes, including cellulases, proteases, gluconases, and chitinases, are generally active against fungi because of the presence of chitin and glucan in their cell wall. Quorum-quenching enzymes include lactonases and deaminases. They found application in disrupting the quorum sensing signal of many Gram-negative plant pathogens, which rely on AHLs-QS for virulence.

Additionally, *B. subtilis* produces a wide range of bioactive polyketides (PKs), including bacillaene, difficidin and macrolatin. PKs are antibiotics with bacteriostatic activity that are able to stop protein biosynthesis in bacteria through a mechanism that is not well-defined (Patel et al., 1995; Zweerink et al., 1987).

In addition to the NRPs previously described (surfactin, iturin, fengycin), *B. subtilis* also produces bacilysin, bacitracin and mycobacillin, non-thiotemplate NRPs which are known to interfere with cell wall assembly by suppressing the peptidoglycan synthesis (Caulier et al., 2019).

An important note should be dedicated to the production of Volatile Active Compounds (VOCs): a variety of chemical compounds such as ketones, alcohols, esters, and sulfur-containing compounds. These VOCs play roles in interspecies communication, antimicrobial activity against pathogens, and plant growth promotion. Their mode of action has not been fully elucidated. However, morphological cell abnormalities have been shown upon the exposition of bacterial and fungi cells to *B. subtilis* VOCs. Moreover, the 2,3-butanediol, resulting from glucose metabolism, was shown to reduce the virulence of *P. carotovorum* (Tahir et al., 2017), while 2-butanone was found to be able to induce plant stress tolerance and promote plant growth (Audrain et al., 2015).

2.4 Regulatory network

Regulation of antimicrobial biosynthesis is a complex and intricate network that connects population density (quorum sensing), the metabolic state of the cell (state transition), and cellular differentiation (Fig. 5)

The biosynthesis of antimicrobial compounds is strictly connected with competence. Competence allows bacteria cells to uptake DNA from the environment. Therefore, a regulation system that integrates the release of antimicrobial compounds with the DNA uptake, allows bacteria to use one signal to control two mechanisms.

Competence in *B. subtilis* is regulated by a quorum sensing system based on the signaling molecule ComX. The membrane protein ComP senses the presence of ComX and activates the phosphorylation of ComA (Turgay et al., 1998). Phosphorylated ComA then activates the transcription of two key genes: *comS*, essential for bacterial competence, and *srfA*, involved in surfactin synthesis. Although surfactin itself is not directly related to competence, its role in inducing cell lysis leads to the accumulation of DNA in the surrounding environment of the bacteria. This extracellular DNA can then be readily taken up by the competent cells. (Stein et al., 2005).

The synthesis of surfactin is also regulated by the product of *phrC* gene, a signalling peptide named as the competence and sporulation stimulation factor (CSF). The cellular import of CSF is facilitated by the oligopeptide permease Spo0K. CSF interacts with the Rap protein, resulting in the loss of phosphatase activity in Rap. This prevents the dephosphorylation of phosphorylated ComA, thereby promoting the transcription of the *srfA* gene and facilitating the synthesis of surfactin. (Hu et al., 2019).

Because ComX and CSF concentrations depend on cell density within the medium, the expression of *srfA* is primarily regulated by cell density. Additionally, the expression of *srfA* is suppressed by the nutritional repressor CodY. This occurs when CodY binds directly to the *srfA* promoter in response to rising concentrations of casamino acids in the growth medium (Serror and Sonenshein, 1996).

Yazgan Karata et al., demonstrated in 2012 that bacilycin follows the same regulatory pathway of surfactin. Mutational analysis revealed that ComA and CSF are, in fact, both involved in the transcriptional regulation of bacilycin.

ComA-P also activates the transcription of *degQ*. The small protein DegQ actively contributes to the phosphorylation of DegU, a master regulator of the transition from a motile cell state to a biofilm-forming state. DegU up-regulates the production of fengycin, iturin, bacillaene, and difficidin (Yu et al., 2023).

The synthesis of antimicrobial compounds is also coordinated by the transition state of growth. In this context, AbrB, Spo0A and σ^H are key regulators. During the logarithmic phase of growth, *spo0H* is repressed by AbrB. When the culture transitions into the stationary phase, levels of phosphorylated Spo0A gradually increase. Phosphorylated Spo0A represses the transcription of *abrB*, resulting in the release of *spo0H* repression (Karata et al., 2003).

The product of *spo0H*, σ^H, is required in the transcription of CSF from *phrC*. So, the repression of AbrB protein by Spo0A-P leads to the expression of CSF and thus indirectly regulates the expression of *srfA*.

AbrB also controls the synthesis of subtilin. Precisely, *spaS* encodes the subtilin precursor, which undergoes post-translational modification by SpaBC before being exported outside the cell by SpaT. Then, the precursor is cut by proteases in its active form. *spaS* is under the control of the a dual component system SpaRK that is regulated by AbrB through the *spo0H* transcription. During the exponential phase, AbrB blocks *spo0H* and, therefore, functions as a repressor of subtilin biosynthesis (Zhang et al., 2022).

Interestingly, antimicrobial compounds that are activated by Spo0A-P are also involved in the antagonism against non-sporulating sister cells (Spo0A-inactive). Sporulation is extremely costly in terms of energy, and Spo0A-active cells use cannibalism as a way to delay sporulation. When levels of intracellular Spo0A-P rise but are still below the threshold for sporulation, bacteria produce two toxins: the sporulation-killing factor (Skf) and the sporulation-delaying protein (Sdp). These two toxins serve as a way to kill sibling bacteria and increase nutrient availability, therefore delaying the sporulation (Rahman et al, 2021).

Regulation of Spo0A is itself extremely complicated and involves many feedback loops. Phosphorylation of Spo0A induces the expression of *spo0H.* Later, σH stimulates the expression of both the genes needed for phosphorylation and the expression of Spo0A. Finally, the high level of phosphorylated Spo0A initiates the expression of genes required for sporulation.

Bacteria employ strategies to protect themselves from the effects of secreted antimicrobial compounds. This can involve either deactivating the active molecules or actively removing them from the cells. For instance, the membrane-bound protein YerP is

implicated in both the secretion of surfactin and providing self-resistance against surfactin in *B. subtilis*.

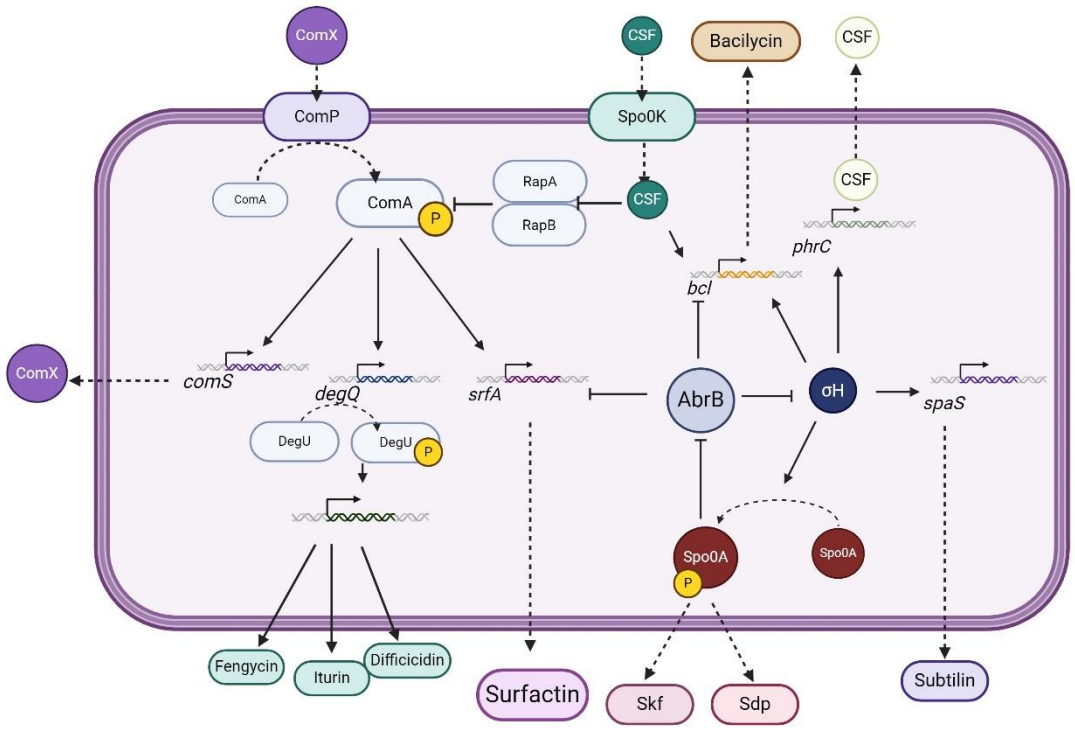

Figure 5- Regulatory network of the synthesis of antimicrobial compounds in B. subtilis. For detailed description see the text.

3 Bacteria social interactions

As unicellular organisms, bacteria have been for long considered independent free-living cells that grow dispersed in the environment. Recent studies have entirely overturned this idea, showing that bacteria have a highly active social life and organize into multicellular communities (Crespi 2001; Dinet et al., 2021). Social interactions among bacteria influence single-cell gene expression, physiology, and survival. At the same time, these interactions have a profound impact on the dynamics and functionality of the entire bacterial community.

In the natural environment, bacteria mono-cultures do not really exist. Instead, bacteria coexist in diverse microbial communities where they exchange information on the state of growth, population density, and metabolic status of the colony through the production, secretion, and detection of chemical compounds in the environment (Taga et al., 2003).

Faust and Raes, in 2012, reviewed how even in the easiest system, consisting of only two species, bacteria can engage in six different types of interactions (Fig. 6) The chemical compounds secreted by one species can be beneficial (public good), indifferent (no effect) or deleterious (toxic) for the other group.

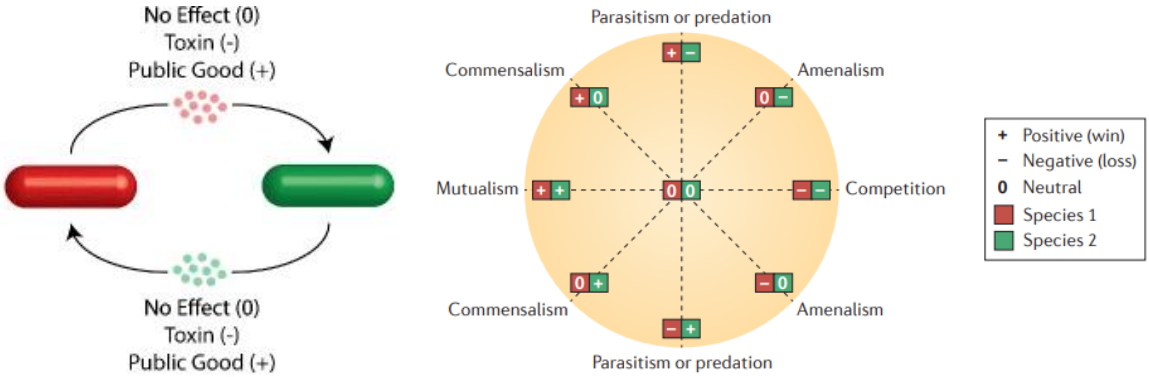

Figure 6 - Bacteria social interactions (A) Activity of the secreted molecules (B) types of interaction. (Faust and Raes 2012)

Positive social interactions include mutualism, when bacteria cooperate to form, for example, multi-species biofilm, or commensalism, when bacteria exchange metabolic compounds that benefit both. On the other hand, loss-win interactions, like predator-prey and host-parasite relationships, involve one partner benefiting at the expense of the other. Amenalism describes situations in which bacteria do not directly compete, but their metabolic compounds can alter the external environment in a way that is not beneficial

for the other species. Lastly, competition is a lost-lost interaction that is observed when bacteria compete for the same niche and resources.

In addition to interspecies communication, bacteria have evolved social behaviours that allow individual cells to function as a social unit. Multicellular lifestyles have emerged independently in different bacterial species and are characterized by inter-cellular communication, division of work, and cell-cell adhesion. Living in communities provides many advantages to bacteria. They can resist environmental stress, acquire more nutrients, protect themselves from predators, and utilize available resources more efficiently (Lyons and Kolter, 2015).

Biofilm formation is the most investigated bacteria social behaviour: bacteria can colonize surfaces by forming three-dimensional and multicellular aggregates embedded in a self-produced matrix. As an alternative to sessile aggregation, bacteria can migrate over a surface in a process known as swarming. This phenomenon allows bacteria to grow and spread simultaneously over a surface, reducing the cell-to-cell competition for nutrients. These collective behaviours involve changes in gene expression while still maintaining genetic identity. Therefore, in many bacteria, these phenomena are regulated by a quorum-sensing mechanism, which serves as a cell-to-cell communication system to regulate cell differentiation. For instance, *B. subtilis* has been intensively studied for its ability to differentiate in several subpopulations. For this reason, it is also called the 'master of differentiation'. For instance, during the early stationary phase, bacteria can differentiate into (i) surfactin producers with competence potential, (ii) matrix producers involved in biofilm formation, (iii) motile cells capable of swarming to explore new niches, and (iv) sporulating cells, which represent a last-chance survival strategy (Rahman et al., 2021).

3.1 Swarming motility

Swarming motility is a coordinated and directional movement of bacteria over a semi-solid surface. In liquid media, bacteria involve flagella rotation into swimming motility which allows individual cells to move independently in the environment, following chemotactic adaptation. (Harshey et al., 2003).

When flagellated bacteria are grown over solid and semi-solid surfaces, they exhibit a wide range of motility, including twitching, gliding, sliding, and swarming. Sliding is the passive spread of bacteria caused by bacteria multiplication and cell division. Twitching

motility is powered by pilus extension and retraction allowing cells to move slowly over a surface. Gliding motility is a type of translocation that does not involve external appendages such as flagella or pili. Sliding, gliding, and twitching are all types of movements on solid surfaces that happen at single-cell levels and are, therefore, defined as individual behaviours. On the other hand, swarming motility is a multicellular movement that involves coordination and synchronization. Being a collective behaviour, swarming is under the control of quorum sensing.

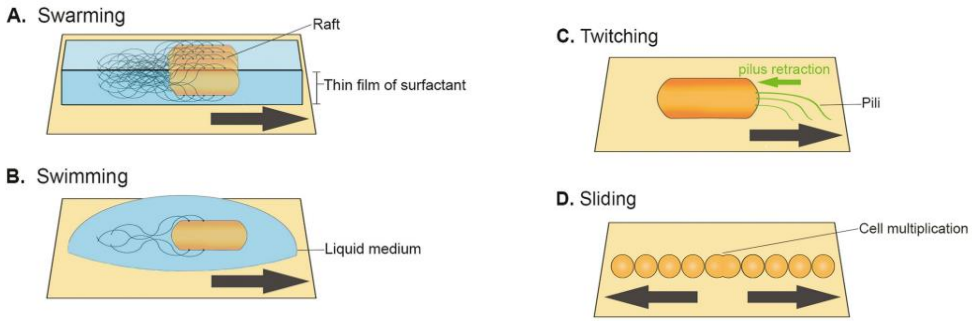

Figure 7- Types of bacteria motilities. See the text for a detailed description (Kearn, 2010)

Swarming is defined as a plastic movement because it is highly dependent on environmental conditions. Availability of nutrients, the type of medium, concentration of agar, and humidity can influence the ability of bacteria to swarm, the swarming pattern, and the duration of the lag phase. For instance, *P. aeruginosa* is not able to swarm on nutrient-rich media while swarming normally occurs on minimal medium (Badal et al., 2021). Alternatively, *B. subtilis* swarms on a wide range of rich media, while *S. enterica* and *Y. enterolitica* require the addition of particular nutrients, including glucose, to swarm (Roy et al., 2021; Young et al., 1999)

In laboratory conditions, the agar concentration plays a crucial role in limiting swarming behaviour. On the basis of the percentage of agar that is suitable to support swarming, bacteria are divided into robust swarmers (up to 3%), temperate swarmers (0.5%-0.8%), and soft swarmers (0.3% - 0.5%). For instance, *Proteus mirabilis* is a robust swarmer, *B. subtilis* is a temperate swarmer while *D. solani*, which swarms on 0.5% agar, is considered a soft swarmer (Partridge and Harshey, 2013).

Environmental conditions also influence the swarming pattern, as the same bacterium can exhibit different patterns on different media. Patterns of bacteria swarming have been reviewed by Kearn in 2010. Briefly, *P. mirabilis* exhibits a characteristic bull's eye swarming pattern; starting from the inoculation point, the swarming front advances, then

stops and starts again, forming characteristic rings. *B. subtilis* instead forms highly ramified tendirls departing from the inoculation point. Alternatively, *PaeniBacillus vortex* forms spiral vortices on the medium while migrating (Fig. 7).

Swarming requires specific conditions and morphological changes. First of all, bacteria require flagella to swarm. While swimming motility is easily supported by a single rotating flagellum, swarmer cells are equipped with several additional flagella (peritrichous) or a second polar flagellum (lophotrichous). When cells transition from swimming to swarming, flagella regulators are upregulated, and cells become hyperflagellated. These modifications enhance the propeller ability of the cell and support swarmers in overcoming surface friction. To ensure that swarmers have a wider area to accommodate additional flagella, swarmer cells undergo morphological changes, cell division is suppressed, and cells appear elongated. This conclusion comes from observations on *P. mirabilis*, which forms short rod cells in liquid culture and long filamentous cells on solid surfaces (Hoeniger et al., 1965).

Peritrichous flagella usually move side-by-side in a clockwise direction allowing bacteria to bundle together, forming cell groups called rafts. Inside the raft, bacteria move in the same direction: each cell tries to move straight but soon touches other cells. These collisions maintain the population in a consistent direction, forming the rafts. Raft formation is very dynamic: cells that join a raft move collectively with the group, while those that detach from the raft lose their motility rapidly. This dynamic process of recruitment and loss implies that there is no specific substance or matrix responsible for maintaining the stability of the raft apart from the flagella themselves (Jose and Singh, 2020).

Surface tension can retain swarming motility; therefore, bacteria usually secrete surfactants (surface active compounds) to reduce the friction between the substrate and the cell. Surfactants are visible as a thin and translucent watery layer that precedes the swarming front. Surfactants are usually glycolipids or lipopeptides and vary from species to species. For instance, *B. subtilis* secrete surfactin, a potent detergent that promotes swarming by releasing surface tension. Surfactin-deficient strains of *B. subtilis* are not able to swarm. However, the addition of external surfactin can restore the swarming motility.

When cells are transferred from liquid culture to solid medium, there is a period of time that precedes swarming, commonly referred to as “swarm lag”. During the swarm lag, bacteria grow as a circular colony on the surface mainly due to sliding (growth-expansion). This phase is required to start biosurfactant synthesis and to reach a population density that allows swarming motility. In many bacteria, swarming is under control of quorum sensing which regulates the production of surfactants and the hyperflagellation of the cells (Kearns, 2010)

3.2 Quorum sensing

Quorum sensing is a cell-to-cell communication system that allows bacteria to coordinate the expression of some genes based on population density. Quorum sensing mechanism can vary between bacteria, but it is generally based on the synthesis, secretion, and detection of chemical compounds defined as “autoinducers” (Ng and Bassler, 2009). By monitoring the external concentration of the autoinducer, bacteria can retrieve information about the population density in their surroundings and modulate social behaviours accordingly. QS systems are present in both Gram-positive and Gram-negative while differing in the nature of the autoinducer. In Gram-negative, QS molecules are diffusible signals that can freely pass the membranes, mainly N-acyl homoserine lactone (AHL) molecules, and directly bind the transcriptional regulators. In Gram-positive, autoinducers are usually peptides that undergo post-transcriptional modification during secretion. Crosstalk between Gram-positive and Gram-negative bacteria via QS signals has been observed in various ecological niches. For example, interspecies communication between Gram-positive *Streptococcus pneumoniae* and Gram-negative *Haemophilus influenzae* has been demonstrated through the production and detection of the same autoinducers for the formation of a multispecies biofilm (Tikhomirova et al., 2013). Conversely, certain *Bacillus* species have been shown to possess the ability to degrade acyl-homoserine lactones (AHLs) produced by gram-positive bacteria, thereby interfering with their intercellular communication. (Anandan et al., 2019).

3.3 Biofilm

Biofilms are microbial communities organized in a self-matrix-produced 3D structure. The matrix represents 90% of the biomass, and it is composed of polysaccharides (EPS), proteins, and extracellular DNA (eDNA) and acts as a stabilizing scaffold for the three-dimensional expansion (Zaho et al., 2023). Bacteria biofilm grows on biotic and abiotic,

solid, and liquid surfaces. Under favourable conditions, the thickness of the biofilm may reach several millimetres.

In the natural environment, biofilms are usually multispecies. These interspecies interactions provide many benefits to the community, including increased mass, increased antibiotic resistance, and enhanced metabolic activity (Sadiq et al., 2021).

Biofilm formation is a multi-steps process. Initially, planktonic cells attach to a surface, this transition is complex and highly regulated as cells growing in biofilms show significant physiological differences when compared to the planktonic phase. This attachment is initially reversible and becomes irreversible when cells form the extracellular matrix and form the microcolonies. Dispersion represents the final stage in biofilm development. During dispersal, microcolonies experience cell death and lysis, accompanied by the active departure of motile bacteria, resulting in the formation of hollow colonies. It is believed that biofilms maintain equilibrium through a balance between growth and dispersal (Sauer et al., 2022)

Biofilm formation and swarming motility are usually mutually exclusive behaviours. Cells typically express genes required either for motility or biofilm production. However, the switch between swarming and biofilm formation is an object of discussion. For instance, flagella are commonly involved in the initial stages of biofilm formation, aiding in reversible attachment, while surface motility is essential for structuring the biofilm architecture. However, motility also plays a role in dispersing bacteria from mature biofilms. Consequently, bacteria may alternate between motility, such as swarming, and biofilm formation at different stages of growth (Verstraeten et al., 2008).

II. AIM OF THE STUDY

The aim of this study is to comprehensively investigate the competitive dynamics between *Bacillus subtilis* MB73/2 and *Dickeya solani* IFB102, two putative antagonistic species naturally inhabiting the plant rhizosphere. Specifically, we aim to:

- investigate the competitive strategies employed by the interacting species focusing on elucidating whether these species engage in indirect competition for resources or actively interfere with each other through the release of antimicrobial compound/compounds.
- elucidate the molecular mechanisms underlying the antagonism, with the aim of understanding the signalling pathways involved.
- evaluate a potential application of *B. subtilis* as biocontrol agent against *D: solani*

By addressing these points, this study aims to advance our understanding of the ecological dynamics within the plant rhizosphere and contribute to the development of sustainable and environmentally friendly strategies for managing of *D. solani* infections in agriculture.

III. MATERIALS AND METHODS

4 Materials

4.1 Strains

4.1.1 E. coli strains

Strain	**Source of reference**
DH5α	Sambrook *et al*., 1989
PSB401	Winson et al. 1998

4.1.2 B. subtilis strains

Strain	**Source of reference**
168	Anagnostopoulos & Crawford, 1961
168 *sfp*+	Prof. M. Obuchowski
MB73/2	Krzyzanowska et al., 2016
MB73/2-*gfp*	Prof. M. Obuchowski
MB73/2 - *sfp*	This work
MB73/2-*spo0A*	This work

4.1.3 D. solani strains

Strain	**Source of reference**
IFB102	Prof. M. Obuchowski
IPO2222	Prof. R. Czajkowski
IPO222-*mCherry*	Prof. M. Obuchowski
D s0432-1	Prof. R. Czajkowski
IFB102 *ΔLysR*	This work

4.2 Primers and Reagents for PCR reaction

Amplified gene	Name	Sequence	Restriction site
sfp	sfp-F	atta**GGATCC**ACGGTTCATGTCTTTCATATC	*Bam*HI
	sfp-R	atta**GTCGAC**GATATAGCATGGGGAATGG	*Sal*I
spo0A	Spo0A-F	atta**GGATCC**TTATTATGCCGCATCTAGA	*Bam*HI
	Spo0A-R	ttat**GTCGAC**GCCACTTCAATTGCATG C	*SalI*
Region 529438-549873	noMB-F	AACCATGTCCTAAACGCT	-
	noMB-R	TGGAAAGAAGCACAAGAAGAG	-
gentamycin	Gent-F	gagaggattcgagAGGACGCGTCAATTCTCG	-
	Gent-R	gtattacaaggctTAACAGATGAGGGCAAGC	-
LysR-Left	LysRLeft-F	cggccagtgaattcgagctcggtacAAGCTTGTTTCGG TGTTG	-
	LysRLeft-R	ttgacgcgtcctCTCGAATCCTCTCGTATTATTTT C	-
LysR-Right	LysRright-F	cctcatctgttaAGCCTTGTAATACGGTCC	-
	LysRright-R	gcatgcctgcaggtcgactctagagCTATTCTAATTCGT TCCGTTG	-

4.3 Enzymes and Reagents

- T4 phage DNA Ligase and buffer for ligase (Thermo Scientific, Lithuania)
- Alkaline phosphatase (Thermo Scientific, Lithuania)
- Lysozyme (Sigma, USA)
- FastDigest™ restriction enzymes and buffers for restriction (*Bam*HI*, Sal*I) (Thermo Scientific, Lithuania)
- Nucleotides dNTPs (dATP, dCTP, dGTP, dTTP) (Thermo Scientific, Lithuania)
- Phanta Max Super-Fidelity DNA Polymerase P505 and buffer (Vazyme, China)
- Q5 High Fidelity Polymerase and buffer (New England Biolabs United Kingdom)
- DreamTaq Green and DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, Lithuania)
- BEBuilder HiFi DNA Assembly and Master Mix (New England Biolabs United Kingdom)
- Methylnitronitrosoguanidine (MNNG) (Sigma, USA)

4.4 Growth and Recovery media

4.4.1 Luria Bertani (LB) agar

Bacto-agar	15 g/l
Yeast extract	5 g/l
Tryptone	10 g/l
NaCl	10 g/l

pH= 7.0

autoclaved for 20 minutes at 121 °C

4.4.2 Luria Bertani (LB) broth

Yeast extract	5 g/l
Tryptone	10 g/l
NaCl	10 g/l

pH= 7.0

autoclaved for 20 minutes at 121°C

4.4.3 TSB medium

Soytone	3 g/l
Tryptone	10 g/l
NaCl	5 g/l
Glucose	2.5 g/l
Dipotassium Phosphate	2.5 g/l

pH= 7.3

autoclaved for 20 minutes at 121°C

4.4.4 B-medium (1x)

$(NH_4)_2SO_4$	15 mM
$MgSO_4$	8 mM
KCl	27mM
Sodium citrate dihydrate	7 mM
Tris/HCl (pH 7.5)	50 mM
KH_2PO_4	0.6 mM
CaCl	2 mM
$FeSO_4$	2,1 µM
MnSO4	10 µM
Glutamic acid	4.5 mM
Tryptophan	0.78 mM
Lysine	0.8 mM
Glucose	0.5% (w/v)

pH=7.0

4.4.5 Minimal Medium A (MMA)

$(NH_4)_2SO_4$	1 g/l
Mg_2SO_4	0.1 g/l
K2HPO4	7 g/l
Citric acid	0.5 g/l
KH2PO4	3 g/l
Glycerol	0.2% (w/v)
pH=7.0	

4.4.6 MSM medium

Glucose	0.5% (w/v)
Casamino acids	0.02% (w/v)
Tryptophan	14 µg/ml
FAC	2.2 µg/ml
MSM salts	1x
pH= 7.0	

4.4.7 MSM salts (5x)

$(NH_4)_2SO_4$	75.5 mM
K_2HPO_4	0.4 M
KH_2PO_4	0.22 M
Trisodium citrate	19 mM
$MgSO_4$ x $7H_2O$	4 mM
pH = 7.4	

4.4.8 Starvation Medium

Glucose	0.5% (w/v)
MSM salts (5x)	1x

4.4.9 PAB (1x)

Peptone	10 g/l
Yeast Extract	1.5 g/l
$NaCl_2$	3.5 g/l
Glucose	1.0 g/l
Beef extract	1.5 g/l
K_2HPO_4	3.7 g/l
KH_2PO_4	1.32 g/l
pH=6.9	

4.4.10 SMPP (2x)

BSA	0.3% (w/v)
Sucrose	2 mM
PAB 4x	25% (w/v)
SMM 2x	50% (w/v)

4.4.11 SMM (2x)

Sucrose	1 M
Maleic acid	4 µM
$MgCl_2$	4 µM
pH= 6.5	

4.4.12 SMMP

SMM	75% (w/v)
PAB (4x)	25% (w/v)

4.5 Antibiotics

		Working concentration			
Antibiotic	**Solvent**	***E. coli***	***D. solani***	***B. subtilis***	**Producer**
Ampicillin	H_2O	100 µg/ml	40 µg/ml	-	Sigma (USA)
Kanamycin	EtOH	-	-	25 µg/ml	Sigma (USA)
Erythromycin	EtOH	-	-	10 µg/ml	Sigma (USA)
Spectinomycin	H_2O	-	-	100 µg/ml	Sigma (USA)
Gentamycin	H_2O	-	20 µg/ml	-	Sigma (USA)

4.6 Buffers

4.6.1 RF1 Solution

CH_3COOK	30 mM
RbCl	100 mM
$CaCl_2$	10 mM
$MnCl_2$	30 mM
Glycerol	15% (v/v)
pH= 5.8	

4.6.2 RF2 Solution

MOPS	10 mM
RbCl	10 mM
$CaCl_2$	75 mM
Glycerol	15% (v/v)
pH= 6.5	

4.6.3 PBS

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4 x $2H_2O$	10 mM

KH_2PO_4	2 mM
pH= 7.4	

4.6.4 TAE Buffer (50x)

Tris pH= 8.3	2 M
Acetic acid	1 M
EDTA	50 mM

5 Methods

5.1 Bacteria media and growth conditions

Bacteria were cultured in Luria broth (LB) medium supplemented with antibiotics when necessary. Growth temperatures were tailored to the requirements of each strain: 28°C for *D. solani* and 37°C for *E. coli* and *B. subtilis*. Cultures were incubated with shaking to ensure efficient aeration. Bacterial stocks were prepared in 25% glycerol, cryopreserved with liquid nitrogen, and stored at -80°C. Fresh plates were prepared every three days from the glycerol stock. *D. solani* plates were kept at room temperature (RT) to preserve the bacteria ability to swarm, as refrigeration compromised this characteristic.

5.2 Swarming motility assay

5.2.1 Swarming motility of D. solani

A single colony of *D. solani* was inoculated in LB medium and incubated overnight with shaking at 28°C. Two microliters of the overnight culture (OD600 ≈ 0.8) of *D. solani* and were inoculated in the center of a B-medium plate (with 0.5% of agar) and incubated flipped for 24h or 48h at 28°C (relative humidity at least 80% saturation). Plates were prepared 1h before the inoculation and dried open for 30 min in a laminar flow chamber. B-medium plates were prepared with different volume of medium (7.5ml, 10ml, 15ml, 20ml and 25 ml) and increasing concentration of medium (0.5x, 1x, 1.5x, 2x and 2.5x). Glucose influence on swarming motility was determined in plates containing 7.5ml of 0.5x concentrated B-medium (0.5% of agar) with increasing concentration of glucose (0, 0.01, 0.05, 0.1, 0.25, 0.3, 0.4, 0.5, 1, 1.5, 2, 3, 3.5, 4 and 4.5% (w/v)).

5.2.2 Swarming motility screening of antagonistic interaction

Single colonies of *D. solani* and *B. subtilis* were transferred in separate LB flasks and cultivated overnight at 28°C and 37°C, respectively. On the day of the experiment, plates containing 7.5ml of 0.5x B-medium with 0.5% of agar, if not differently indicated, were prepared one hour prior to inoculation. The plates were dried for 30 minutes. *B. subtilis* and *D. solani* were inoculated on the same swarming plate at a distance of 1.5 cm. Plates were incubated at 28°C with the lids facing downward (relative humidity at least 80% saturation). The day after, the swarming interaction pattern was visualized with high resolution camera it is Optilia W30x-HD in each plate. Plates were scanned to store

images. Each experiment was repeated three times, using mono-species swarming plates as controls.

5.3 Detection of AHLs released in B-medium agar plates

We developed a fast-screening method for the detection of N-Acyl homoserine lactones (AHLs) in agar plates based on the method previously presented by Jafra et al. (2004). *E. coli* pSB401 was used as a biosensor for detection of AHLs due to its high level of bioluminescence mediated by the presence of N-3-(Oxohexanoyl)-L-homoserine lactones. A single colony of *E. coli* pSB401 was inoculated in 5 ml of LB supplemented with 20 μg/ml of tetracycline and incubated overnight at 37°C. The day after, the culture was diluted to OD600 ≈ 0.1 and incubated for 5 hours. Swarming motility plates were examined to determine the presence of AHLs. Thirteen holes of ∼9 mm diameter were cut from each plate by using a flamed cork borer. The circular agar pieces were collected at 1 cm away from each other, starting from the inoculation point and proceeding in the four directions (above, below, left, right) up to 3 cm away from the point of inoculation. The circular agar samples were transferred directly to a sterile 96 wells plate and each well was inoculated with 150 μl of diluted suspension of the indicator strain (OD600 ≈ 0.2). Plates were incubated overnight at 37°C, the growth temperature not permissive for *D. solani*. Emission of chemiluminescence was detected with the ChemiDoc XRS+ system (BIO-RAD).

5.4 Cell Free supernatant (CFS)

5.4.1 CFS Preparation

B. subtilis MB73/2 was inoculated in 25ml of LB and incubated overnight at 37°C with shaking. The culture was transferred into 50ml falcon tubes and centrifuged (8000 g, 15 min, 4 °C). Supernatant was collected by sterile syringe with needle and then filtered with a sterile polyethersulfone (PES) membrane filter with pore size 0.22 μm. The CFS was used directly or concentrated after freeze-drying

5.4.2 CFS freeze-drying

The supernatant of *B. subtilis* MB73/2 and pure LB were aliquoted in 2 ml Eppendorf tubes and frozen at −80 °C for 24 h. The samples were lyophilized using the Labconco FreeZone® TriadTM Freeze Dry System. The lyophilizer was set at -40°C for 5h, time required to reach this temperature. Lyophilization program was optimized for being

performed in 2 ml Eppendorf tubes and using a volume of 1.5 ml. Aliquots were then rehydrated in sterile LB prior to use.

T(C°)	TIME (HH:MM)	VACUUM (MMBAR)
-40	1:00	OFF
-40	0:30	0.04
-25	4:00	0.04
10	6:00	0.01
10	1:00	OFF

5.5 Determination of the antibacterial activity of MB73/2 CFS

5.5.1 Inhibition of growth

D. solani IFB102 was inoculated into LB medium and cultivated overnight at 28°C with shaking. Bacteria culture was washed in 1ml, 0.5ml and 200 µl of PBS (4500 g, 10 min, 4 °C). Lyophilized CFS was resuspended in LB to obtain 1x, 10x and 25x concentrated supernatant. A bacteria suspension at OD600=0.03 was added to media containing increasing concentration of MB73/2 CFS and to LB as control. OD600 was measured for 8 hours, an end point sample was collected after 18 hours. The experiment was performed twice in triplicates.

5.5.2 Inhibition of swarming motility

D. solani IFB102 was inoculated into LB medium and cultivated overnight at 28°C with shaking. Two µl of overnight culture were inoculated at 1.5 cm distance from 20 µl of MB73/2 CFS spotted on a swarming plate. Plates were incubated overnight at 28°C (relative humidity 80%). Plates were visually inspected to determine the direction of swarming of *D. solani* IFB102 in relation to the presence of MB73/2 CFS. If the cells migrated toward the MB73/2 CFS, it was noted as ‘no antagonism. Conversely, if the majority of cells migrated away from the CFS, it was recorded as ‘antagonism’. The direction of swarming was determined based on visual inspection of the plates and recorded as the endpoint of the experiment. The experiment was performed in triplicates. LB was used as control. At the same time, swarming plates were prepared supplementing the 7.5ml of 0.5x B-medium with 1ml of MB73/2 CFS. Two µl of overnight culture of

IFB102, were dropped at the center of the plate. Plates were incubated according to the swarming motility assay protocol (5.2). Following incubation, plates were visually inspected to determine *D. solani* IFB102 swarming ability and swarming pattern in the presence of MB73/2 CFS.

5.6 *B. subtilis* MB73/2 mutagenesis

5.6.1 Chemical mutagenesis

B. subtilis MB73/2 was cultured overnight in LB according to its specific conditions of growth (5.1). The day after, the culture was refreshed in new LB and cultured until mid-log phase (OD600=0.3-0.6). One ml of bacterial culture was collected and centrifuged (4500 g, 10 min, 4 °C). The resulting pellet was washed with PBS buffer for three times (4500 g, 10 min, 4 °C). After washing, the pellet was resuspended in 100 µl of PBS. The chemical mutagen MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) was added to the bacterial suspension at a final concentration of 20 µg/ml. Samples were incubated at 37°C for 30 minutes. Then, 30 µl of culture were diluted in 1ml of PBS. Bacteria were washed three times in PBS buffer to remove residuals of the MNNG mutagen. Mutants were serially diluted and plated on LB agar plates. Plates were incubated overnight, and the obtained mutant colonies were transferred to new LB agar plates and incubated overnight at 37°C. Obtained mutants were used for the downstream screening.

5.6.2 Transposon mutagenesis

The random mutagenesis of *B. subtilis* MB73/2 via TnYLB-1 transposon insertion was carried out in accordance with the method described by Breton et al. (2006). Initially, the plasmid pMarB was introduced into MB73/2 through protoplast electroporation (5.9.1). Cells were resuspended in the SMMP recovery medium and upon overnight incubation serial dilutions were plated on LB agar plates supplemented with kanamycin (25 µg/ml). Plates were incubated at 46°C for 10h. Obtained colonies were picked and transferred on fresh LB agar plates supplemented with kanamycin (25 µg/ml) and then duplicated on plates containing erythromycin (10 µg/ml). Plates were incubated overnight at 30°C. Transformants that were sensitive to kanamycin and not to erythromycin contained the correct insert and were used in the downstream screening.

5.6.3 Selection of the mutants

D. solani IFB102 was grown overnight in LB according to the standard method (XXX). Overnight culture was refreshed in new LB and growth was extended until early logarithmic phase (OD600=0.2). To prepare the top agar, LB medium containing 0.5% agar was freshly made on the day of the experiment. Ten ml aliquots of top agar were then prepared, and 100 µl of the refreshed culture of *D. solani* IFB102 was inoculated into each aliquot when the temperature was approximately 40°C. The inoculated top agar was then poured onto LB agar plates and allowed to dry under the hood until solidification. Colonies of MB73/2 mutants were transferred onto the top agar using a sterile tip, and the plates were incubated for 24 hours. Following the initial observation at 24 hours, the incubation period was extended to 48 hours. Mutants that showed no inhibition of growth against *D. solani* were selected for the swarming motility antagonistic assay (5.2)

5.6.4 Production of biosurfactants: oil collapse test

The oil-drop test aims at evaluating the presence of biosurfactants in the supernatant of *B. subtilis* MB73/2 mutant strains. Initially, MB73/2 mutant strains were cultured overnight in 5ml of LB at 37°C with shaking. Subsequently, 1 ml of the cultured broth was centrifuged at 10000 g x 10 minutes at 4°C, and the resulting supernatant was collected for the oil-drop test. To perform the test, 10 ml of sterile water was poured into a Petri plate. A drop of 20 µl of mineral oil was carefully added to the water surface. Following this, 5 µl of the supernatant obtained from the MB73/2 mutant strains was spotted into the oil. If biosurfactants are present in the supernatant, the oil drop will be rapidly disrupted within seconds. Conversely, if no biosurfactants are present, the supernatant will remain on the top of the oil surface without causing any disruption. The supernatant of MB73/2 was used as positive control, and the supernatant of *B. subtilis* 168 was used as negative control.

5.7 *D. solani* and *B. subtilis* co-culture

For co-cuture experiments strains harbouring either a fluorescent dye or antibiotic resistance cassette were needed. Due to the difficulty in transforming IFB102, the experiments were conducted with strain mCherry-marked IPO2254 (from now on referred as IPO2222-*mCherry*) harbouring the ampicillin resistance cassette and strain GFP-marked MB73/2-*gfp* harbouring the spectinomycin resistance cassette.

5.7.1 Co-culture in liquid medium

A single colony of MB73/2-*gfp* was inoculated into 100 ml of LB without antibiotics and incubated overnight at 37°C with shaking. Simultaneously, a single colony of IPO2222-*mCherry* was inoculated into 100 ml of LB medium without antibiotics and incubated overnight at 28°C with shaking. After overnight incubation, both bacterial cultures were refreshed in 100 ml of fresh LB medium and incubated until reaching the early exponential phase, as determined by an OD600 of 0.2.

Next, 100 µl of the refreshed cultures were transferred into 100 ml of fresh LB medium and incubated again until reaching an OD600 of 0.2. An aliquot of the culture from each bacterium species was inoculated into fresh LB to achieve an OD600 of 0.05. Subsequently, 100 ml of IPO2222-*mCherry* culture at OD600 of 0.05 was equally mixed with MB73/2-*gfp* culture at OD600 of 0.05. As a control, mono-cultures were mixed with an equal amount of LB medium.

The co-culture and the control mixtures were then incubated at 28°C for 8 hours. Samples were collected every 60 minutes, and serial dilutions were plated on LB agar supplemented with ampicillin for selection of IPO2222-*mCherry*. The results were expressed as a growth curve based on the colony-forming unit (CFU) count per millilitre. After 4h from the starting of the co-culture, 2 µl were spotted on a swarming plate (5.2), and the plate was incubated overnight following the indications previously described. The presence of *D. solani* in the co-swarming pattern was detected using the Leica HCL LSI microscope (5.7.3).The day after, a sample was collected from the liquid co-culture at the 18-hour time point and plated on selective medium to confirm the survival of *D. solani* after the overnight incubation.

5.7.2 Co-culture on solid medium

B. subtilis MB73/2-*gfp* and *D. solani* IPO2222-*mCherry* were cultured independently following the previously described method (5.1). The following day, the cultures were refreshed in 100 ml of fresh LB medium and grown until reaching the early exponential phase (OD600=0.2). From each culture, an aliquot of 100 µl was carefully collected and mixed by pipetting to prepare a 1:1 ratio mixture. Additionally, a 1:2 ratio mixture was prepared by combining 100 µl of MB73/2-*gfp* culture with 200 µl of IPO2222-*mCherry* culture. Subsequently, two microliters of each mixed culture were spotted at the center of a swarming plate containing 7.5 ml of 0.5x B-medium with 0.5% agar. The plates were

then incubated overnight at 28°C with 80% relative humidity. Swarming pattern and species distribution were observed under Leica HC LSI (5.7.3).

5.7.3 Leica HC LSI observation

The distribution pattern of the two strains was analysed using a Leica HCL LSI microscope. To detect IPO2222-*mCherry* strain (λex = 589 nm and λem = 610 nm) and MB73/2-*gfp* strain (λex = 475 nm and λem = 509 nm), lasers 488 and 561 were activated, respectively. Optics 1x and 5x were employed for imaging. Image acquisition and analysis were performed using the Leica Application Suite (LAS AF). Acquisition settings including gain, pinhole, and offset were adjusted for each measurement. The image format was set to 1024x1024 with a speed of 400Hz.

5.8 Screening for antagonistic interaction on potato slices

The screening for the ability to attenuate potato tissue maceration by *B. subtilis* strains was conducted following the method outlined by Jafra et al., 2006, with some modifications. Initially, potato tubers were surface sterilized using 5% sodium hypochlorite for 10 minutes, followed by rinsing twice with sterile water. After air-drying for 2 hours, the tubers were sliced into 1.5 cm thick slices. Using a sterile cork borer, three wells measuring 9 mm in diameter and 10 mm in depth were made in each slice. *B. subtilis* and *D. solani* strains were cultured overnight according to the previously established protocol (5.1) and refreshed in new LB medium the following morning. Subsequently, the wells were filled with 50 µl of a mixture containing equal parts (1:1 ratio) of *B. subtilis* and *D. solani* strains at OD600 of 0.1. Control slices were inoculated with either water or mono-cultures of *B. subtilis* and *D. solani*. The potato tuber slices were then placed in sterile 25cm glass plates filled with 10ml of water to create a moist environment. Plates were incubated at 28°C for 72h. The diameter of rotting tissue was measured.

5.9 *B. subtilis* transformation

5.9.1 Protoplast electroporation

A single colony of *B. subtilis* MB73/2 was inoculated into 5ml of PAB and incubated overnight at 37°C with shaking. A refreshed culture was prepared in 20ml of PAB and incubated until reaching late exponential phase (OD600=1.7-2). The culture was centrifuged (4500 g, 10 min, 4 °C) and the pellet resuspended in 1ml of SMPP medium. This suspension was then subjected to incubation at 37°C with shaking in the presence of

lysozyme (10 mg/ml). After digestion, the culture was centrifuged at 5200 g for 5 minutes at 4°C, and the resulting pellet containing the protoplasts was washed twice in cold SMMP without PAB, each time in a final volume of 200 µl. Subsequently, the pellet containing the protoplasts was resuspended in 200 µl of 10% glycerol and washed twice in 10% glycerol. Finally, the pellet was resuspended in 120 µl of 10% glycerol. Two microliter of plasmid DNA were added to the protoplast suspension at a concentration of 40 ng/ml and incubated on ice for 30 minutes. The whole mixture was transferred into cold 0.1cm electroporation cuvettes (BioRad Gene Pulser) and the electroporation was performed using the Biorad MicroPulser Electroporator. Protocol used voltage 1.8kV with 2.5 ms pulse width. One milliliter of cold SMMP recovery medium was added immediately into the cuvette. The suspension was transferred into a falcon tube and incubated overnight at 37°C. Subsequently, 100 µl of the culture was plated on LB agar with antibiotic and incubated overnight at 37°C. Selected colonies were transferred to new plates and the proper integration of the plasmid was checked by PCR. Obtained transformants were used in downstream applications.

5.9.2 B. subtilis transformation in MSM medium

A single colony of *B. subtilis* MB73/2 was inoculated in MSM medium without antibiotic and incubated overnight at 37 °C with shaking. The day after, the culture was diluted 1:10 in fresh MSM medium and incubated at 37 °C with shaking for 3 hours. Then, the culture was diluted 1:1 with starvation medium and further incubated for 2 hours at 37 °C with shaking. Following the starvation period, 1 µg of DNA was added to 100 µl of the cell suspension, and the mixture was incubated at 37°C with shaking for 30 minutes to facilitate DNA uptake. To induce phenotypic resistance expression, the suspension was then diluted 1:4 in LB medium and incubated for 45 minutes at 37°C with shaking. At the end of the incubation, cell suspension was plated on previously prepared LA agar plates supplemented with proper antibiotic for phenotypic selection. Plates were incubated overnight at 37 °C.

5.10 *D. solani* transformation

5.10.1 Preparation of D. solani competent cells

A single colony of *D. solani* was spread on a TSB agar plate and incubated overnight at 28°C. After 48 hours, all bacteria colonies were scraped from the plate and resuspended in 1ml of 10% sterile glycerol solution. The cell suspension was washed in 10% glycerol

(8000g x 5 min at 4°C). After each washing step, cells were resuspended in a lower volume (1ml, 0.5ml, 0.25ml and 20-30µl). Cells were stored on ice and use immediately for electroporation. into cold 0.1cm electeroporation cuvettes (BioRad Gene Pulser) and the electroporation was performed using the Biorad MicroPulser Electroporator

5.10.2 D. solani electroporation

Competent cells (20-30µl aliquots) were mixed with 1µg of purified plasmid DNA and incubated on ice for 1 hour. The cell-DNA mixture was then electroporated using a Biorad MicroPulser Electroporator at 2.5 kV for 1-2 seconds. Immediately after electroporation, 500 µl of cold LB media was added for cell recovery, followed by incubation at 28°C for 1-2 hours. Transformed cells (100µl) were plated onto LB agar plates with antibiotic and incubated for 48 hours.

5.11 Molecular cloning

5.11.1 Plasmid and Chromosomal DNA isolation

Plasmid isolation was conducted using commercially available DNA isolation kits, namely the Plasmid Mini or EXTRACTME PLASMID DNA kits (A&A Biotechnology, Poland), as per the manufacturer's instructions. Genomic DNA isolation from bacteria cells was performed using the Genomic Mini DNA isolation kit (A&A Biotechnology, Poland), following the manufacturer's instructions. The concentration and purity of DNA samples were assessed using a ND-1000 spectrophotometer (NanoDrop, USA) and associated software. Two microliters of DNA suspension were used for the analysis.

5.11.2 Amplification of DNA fragments by Polymerase chain reaction PCR

Plasmid or chromosomal DNA has been used as template for the reaction. Reactions have been performed in a final volume ranging from 10-50 µl. Following components have been added to the reaction mix: (i) primers (4.2) to the final concentration of 1 µM; (ii) buffer for the polymerase 1x; (iii) nucleotides dNTPs to the final concentration of 0.2 µM; (iv) sterile, filtered, DNase-, RNase-, protease-free H_2O; (v)1 Unit of polymerase per reaction. Q5 (NEW ENGLAND Biolabs) and PhantaMax (Vazyme) high fidelity polymerases have been used for the amplification of genes and gene fragments subsequently used for cloning procedure. DreamTaq (Thermo Fisher Scientific) polymerase has been used for evaluation of proper cloning of genes or gene fragments and integration. The PCR reactions have been performed with C1000 Thermal Cycler

(Bio-Rad, USA). The protocol for PCR was adjusted to the polymerases manufacturer's instructions.

5.11.3 Enzymatic digestion

Plasmid DNA, amplified genes or gene fragments have been digested by restriction enzymes prior cloning procedures. Reactions have been performed in a final volume ranging from 20-50 µl and with reaction time ranging from 5-60 minutes according to manufacturer's instructions for each enzyme. Following components have been added to the reaction mix: (i) buffer for restriction enzymes 1x; (ii) sterile, filtered, DNase-, RNase-, protease-free H_2O; (iii)1 µl of each FastDigest restriction enzymes (ThermoFisher) per reaction; (iv) 1 µg of template DNA.

5.11.4 DNA Purification and Agarose Gel DNA electrophoresis

PCR products and DNA fragments resulting from enzymatic digestion were purified using the Clean-up DNA purification kit (A&A Biotechnology, Poland), following the manufacturer's instructions. Subsequently, the purified DNA fragments were subjected to electrophoretic separation on 1-1.5% agarose gel in 0.5x TAE buffer using the horizontal electrophoretic apparatus Mupid-One (ADVANCE, Japan). Prior to loading onto the gel, DNA suspensions were mixed with DNA loading buffer in a 9:1 ratio. Electrophoretic runs were conducted at 100V for 24-35 minutes. After electrophoresis, the gel was stained by incubating in a 5 µg/ml ethidium bromide solution for approximately 15 minutes. Stained gel was visualized under UV lights at a wavelength of 354 nm using the Gel Doc™ XR System (Bio-Rad, USA) and Quantity One software. The size of DNA fragments was determined by comparing them with bands of DNA size markers.

5.11.5 T4 DNA ligation

Digested plasmid underwent dephosphorylation through incubation with alkaline phosphatase (Fermentas) under conditions specified by the manufacturer's instructions. Subsequently, the linearized plasmid (vector) and PCR products (inserts) were purified using the DNA purification Clean-up kit (A&A Biotechnology, Poland). For the ligation procedure, the purified linearized plasmid and PCR products were combined in a proportion of 1:3 in a final reaction volume of 30 µl. The ligation reaction was carried overnight at 4°C. The reaction mix included: (i) buffer for 1x T4 phage DNA Ligase, (ii)1

unit of T4 phage DNA Ligase, (iii) and sterile, filtered, DNase-, RNase-, protease-free water.

5.11.6 Gibson Assembly

For the construction of deletion strains, we preferred Gibson Assembly. The vector was constructed in silico using the NEBuilder Assembly Tool. The pUC19 plasmid was cut with restrictions enzymes (4.2) and purified. Following this step, 500bp before and after the gene of interest and the resistant cassette were amplified by PCR using overlapping primers designed by the software. PCR products were checked on gel electrophoresis and purified before assembly. The reaction mixture was prepared in a final volume of 10 µl containing a total DNA amount of 50 ng. The following formula was used to estimate the amount of DNA per each fragment:

- Plasmid DNA= plasmid length / plasmid length + 3 (∑inserts length) x 50ng
- Insert DNA = 3 x insert length / plasmid length 3 (∑inserts length) x 50ng

The reaction mix included: (i) linear plasmid, (ii) inserts, (iii) sterile, filtered, DNase-, RNase-, protease-free water, (iv) 1x Gibson Assembly Master Mix (NEBuilder). The mixture was incubated in a thermocycler at 50°C for 60 min. Following incubation, samples were used for transformation or stored at -20°C.

5.11.7 Preparation of E. coli competent cells

E. coli DH5α cells were cultured in LB medium overnight at 37°C with shaking. Subsequently, the overnight culture was diluted 1:100 into 100 ml of fresh LB medium and incubated at 37°C with shaking until reaching an optical density OD600= 0.5. The culture was then centrifuged at 3800 g for 10 minutes at 4°C, and the resulting bacterial pellet was resuspended in 20 ml of cold RF1 solution. After a 5-minute incubation at 4°C, the suspension was centrifuged again, and the bacterial pellet was resuspended in 4 ml of RF2 solution. Following another 5-minute incubation at 4°C, 50 µl aliquots of the cell suspension were transferred to cold 1.5 ml Eppendorf tubes and immediately frozen in liquid nitrogen. The obtained competent cells were stored at -80°C for future use.

5.11.8 Transformation of competent cells

To 50 µl of the *E. coli* DH5α cell suspension, 1 µg of plasmid DNA was added, followed by a 30-minute incubation on ice. The mixture was then subjected to a thermal shock by

incubating at 42°C for 3 minutes. Subsequently, 1 ml of LB medium without antibiotic was added, and the suspension was incubated at 37°C with shaking for 1 hour to allow for phenotypic resistance expression. The bacterial suspension was plated onto LB agar plate supplemented with antibiotic for selection and incubated overnight at 37°C. Purified plasmid DNA obtained from transformants was used for downstream applications.

5.12 Data analysis

5.12.1 NGS data analysis

The microbial genomes of the nine mutants of MB73/2 and of IFB102 and IPO2222 were sequenced by Genomed on the MiSeq platform (Illumina). Genomed performed the NGS sequencing and bioinformatic analysis consisting of *de novo* assembly of reads into contigs. Cutadapt version 3.0 was used to remove adapter sequences, low-quality bases, and other artifacts from the raw sequencing reads. SPAdes version 3.14.1 was utilized for de novo assembly of the filtered reads into contigs. Contigs shorter than 500 base pairs and those with low coverage were removed and FastQC was employed to assess the quality of the sequencing data after filtering. Per each sequenced genome between 32 and 37 filtered contigs were generated with of a total length of around 4.17 Mb. Filtered contigs were mapped on the reference genome of *B. subtilis* 168 (NC_000964) or *D. solani* IPO222 (CP015137) using ProgressiveMauve and gaps were closed using the GapFiller version 1.11.

5.12.2 Statistical analysis

The Shapiro-Wilk test was utilized to assess the normal distribution of the data. Test for variance homogeneity was conducted using the Fisher-Snedecor test. Pairwise differences were assessed using a two-tailed Student's t-test. In cases where the variance was not homogeneous, the Welch's t-test was employed. If the data did not follow a Gaussian distribution, the Wilcoxon test was used. Experiments were conducted with at least two biological replicates and three technical replicates. Charts and statistical analysis, including the tests mentioned above, were performed using GraphPad.

IV. PRELIMIARY RESULTS

6 Interaction between *B. subtilis* MB73/2 and *D. solani* IFB102 on a semi-solid surface

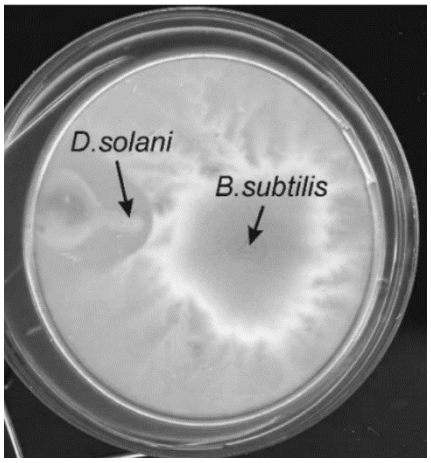

Figure 8 - B. subtilis and D. solani swarming interaction on synthetic medium. Arrows indicate inoculation point (Bikowski M., unpublished).

B. subtilis MB73/2 is an environmental strain isolated form the rhizosphere of soil-grown potato (*Solanum tuberosum L.*) in Zulawy area (Poland) as part of a previous project on the biocontrol potential of *B. subtilis* against a wide range of plant pathogens. MB73/2 was selected from over 600 different isolates of bacteria from the *Bacillus* genus due to its ability to inhibit phytopathogens of genera *Dickeya* and *Pectobacterium* and to colonize potato tuber rhizosphere following seed tuber bacterization (Krzyzanowska et al.,2012). The ability of *B. subtilis* MB73/2 to interact with *D. solani* sp. has been tested on the *D. solani* IFB102 strain, isolated in Poland from infected potato tubers. The interaction between the two species was observed in laboratory conditions by triggering swarming motility according to the methods previously described in literature (Morales-Soto et al., 2015). After inoculation of both strains on the same Petri plate containing a semi-solid synthetic medium, the *Dickeya solani* colony was entirely translocated from the point of inoculation to the edge of the plate, a phenomenon that suggests a strong antagonism between the interacting species. (Fig. 8) The observed phenotype led us to the hypothesis that *B. subtilis* MB73/2 can produce an unidentified antimicrobial compound/compaunds with strong activity on *D. solani* sp. (Bikowski M., unpublished data).

Further repetitions of the experiments have consistently demonstrated that the observed phenomenon is highly dependent on the experimental settings. In particular, to reproduce an effective and directional movement, both species need to be able to act-as-a-community and, consequently, swarm effectively. In the absence of coordinated movement, *Dickeya solani* is unable to successfully escape from the antagonistic effects of *B. subtilis*. Thus, to better understand the intricate social dynamics at play, a lot of effort was dedicated to the optimization of *D. solani* swarming motility under laboratory conditions.

V. RESULTS AND DISCUSSION

7 Optimization of *Dickeya solani* swarming motility assay

7.1 Influence of medium type, time of drying and humidity at incubation on swarming motility

Triggering swarming motility under laboratory conditions can be challenging due to the simplicity of the experimental settings that fail to account for the complexity of the natural environment. Changes in the environment can be difficult to predict and control, making it challenging to reproduce experimental results reliably.

Most publications concerning bacteria swarming motility assays provide general information regarding swarming motility of *D. solani* on synthetic medium containing 0.5% agar. Following these indications for swarming assays, the ability of bacteria to move on the surface appeared to be limited and the swarming pattern was not stable among the replicates. The environmental strain *D. solani* IFB102 is particularly sensitive to the conditions of swarming and requires precisely controlled settings in terms of medium composition and volume, percentage of agar, time of plate drying, temperature and humidity at incubation. (Gatta et al., 2022)

It was expected that the ability of bacteria to move effectively will depend on the available nutrients (energy source) and medium type. Therefore, we investigated the ability of *D. solani* IFB102 to move in a coordinate and directional manner on an energy-rich and on a synthetic minimal laboratory media. Bacteria were grown to the early exponential phase and inoculated at the centre of a swarming plate containing 7.5ml of Luria Bertani (LB) supplemented with 0.5% of agar, an energy-rich semi-solid medium which is known to effectively support growth and swarming motility of Gram-negative and Gram-positive bacteria.

However, at 48h upon inoculation, the swarming movement of *D. solani* appeared to be strongly inhibited on LB agar plates and the bacteria presence in the plate was confined to the area of inoculation, reasonably limited to the colony growth (Fig. 9 A and B).

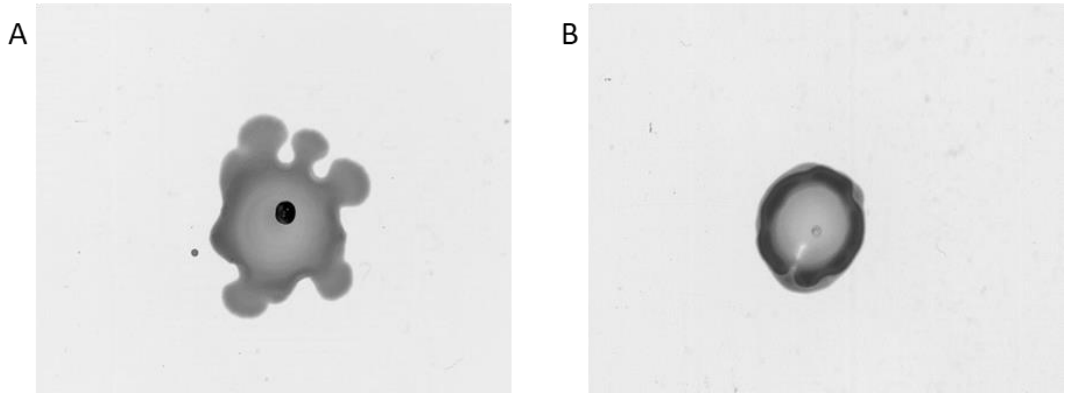

Figure 9 - Swarming of D. solani IFB102 on plates containing 7.5 ml of nutrient rich media (A) LB or (B) NB with 0.5% of agar.

This evidence suggested that nutrient depletion could be a prerequisite for swarming behaviour. Therefore, we repeated the swarming motility assay on the synthetic minimal medium, B-medium, solidified with 0.5% agar. In these conditions, bacteria were capable of swarming faster, and the swarming behaviour was observed for most replicates; nevertheless, their swarming pattern was still not stable.

Swarming phenotype is highly sensitive to environmental conditions and the experimental success and reproducibility strongly depend not only on the nutrient composition and agar type but also on the standardization of the protocol settings that can affect the surface moisture, including, among others, seasonal changes at the stage of media pouring (Morales-Soto et al., 2015). Over the four years of the project, we could determine that seasonal variability of air-humidity in the lab can influence the assay, making swarming more efficient in summer, due to the humid-air, and less stable in the dry-air of winter. This motivated us to explore the best laboratory conditions to obtain a stable and reliable swarming phenotype of *Dickeya solani*.

Bacterial movement on a solid surface is affected by the wetness of the medium. Water surface tension is a key determining factor for bacterial swarming behaviour as it influences the force required for cells to deform the air–water interface and spread on a surface (Yang A; 2017). Consequently, the time of plates drying after pouring the melted agar and the humidity at incubation can affect the thin liquid phase present on the solid surface that is required for bacteria to swarm and can directly interfere with the ability of bacteria of exhibiting swarming behaviour.

To assess the influence of humidity on swarming phenotype of *D. solani* we performed motility assay on synthetic medium while varying the time of plate drying and the

humidity at incubation. To reduce the effects of room-humidity levels in the lab and the seasonal fluctuations, an external humidifier was used during the time of plate drying and plates were incubated in a humidity-controlled incubator.

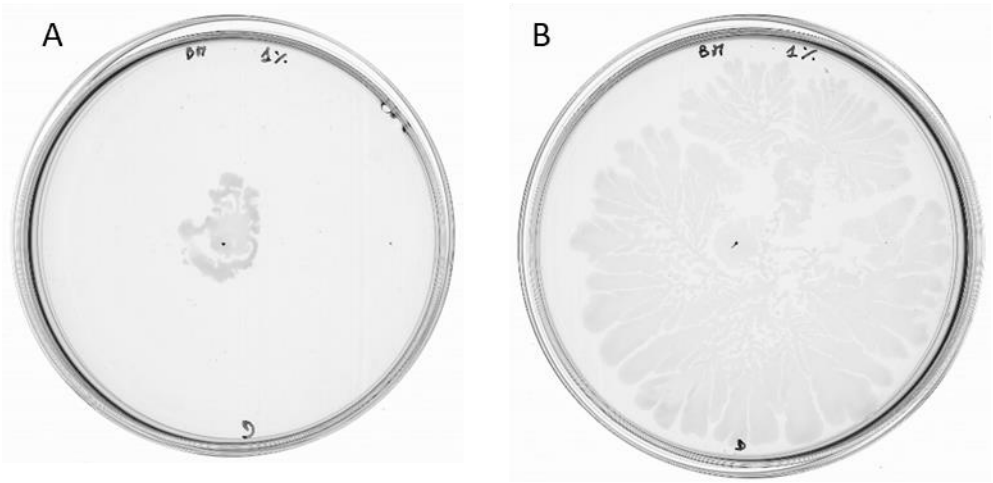

Figure 10 - Swarming of D. solani IFB102 on 7.5mL of B-medium with 0.5% of agar incubated at 50% (A) and 80% (B) of humidity at incubation.

Prior to incubation, the medium must be dry enough to prevent swimming motility and still wet to allow bacteria migration. Successively, the environment during incubation needs to be sufficiently humid to not dry out the medium during the assay and relatively dry to avoid artificial surface spreading. Therefore, we monitored swarming of *D. solani* while increasing the humidity at incubation in the range of 50% to 80% (Fig. 10). Swarming occurred at all tested conditions with a higher humidity at incubation resulting in a faster spreading over the surface.

Accordingly, the time of plate drying affects the swarming velocity, with swarming being extremely limited on plates dried for more than 30 min. Therefore, the optimal drying time was established to be less than 30 min (Fig. 11) with the humidity at incubation fixed at 80%. In principle, modifying the drying time resulted to be the simplest solution to respond to seasonal humidity fluctuations and changes in the hood flow; the precise drying time was adjusted between 20 and 28 minutes according to these external changes. In summary, a higher humidity at incubation gives more space for extending the time of plates drying before inoculation but constant humidity monitoring inside and outside the incubator is required. Minor changes of the surface wettability can significantly impact the reproducibility of the assay and the experimental settings in terms of humidity should

be tested and calibrated in each laboratory before attempting motility assays.

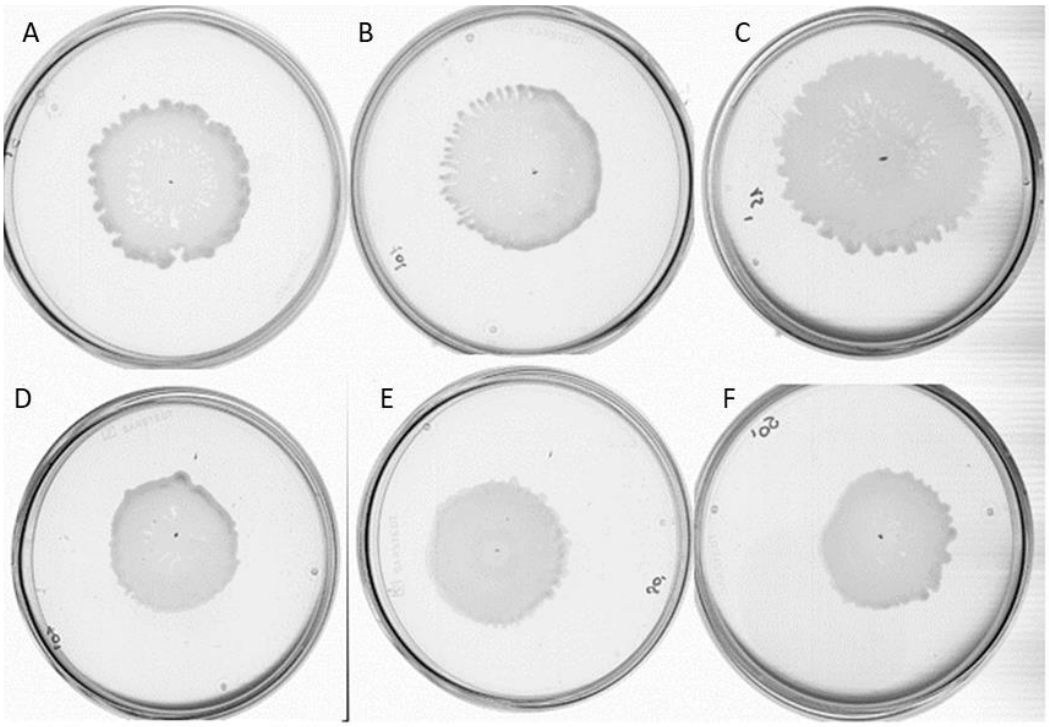

Figure 11- Swarming motility of D. solani IFB102 on 7.5mL B-medium with 0.5% of agar and different drying time: 15min (C), 20min (B), 30min (A), 40min (d), 45min (E), 50min (f). Plates were incubated at 80% of humidity

As the humidity represents a crucial factor for swarming motility, it is not a surprise that the type of plate and plate aeration can influence the moisture of the agar and the success of the experiment. We tested swarming motility on Petri plates of different diameters (35 mm, 60 mm, 90mm) and producers (Starstedt and JSHD), with and without vent. Our experimental settings found the best application on the 92 x 16 mm Starstedt Petri dishes. JSHD plates without ventilation, retain too much liquid that results in an excess of moisture and a switch to swimming motility, while ventilation prevents the excess of condensation but does not support enough moisture for swarming to occur. We used the Starstedt Petri dishes as a balanced standard for our experiments because they better support the swarming.

7.2 Influence of medium volume on swarming motility and quorum sensing

Swarming motility assays are usually performed on plates containing 20-25 ml of medium. We noticed that on plates containing a lower volume of medium bacteria had a better chance of showing swarming behaviour. Therefore, we wanted to verify whether the volume of medium in the plate, and consequently the surface thickness, could

influence the swarming pattern. Motility assay was performed on plates containing increasing volume of medium, in the range of 5ml to 25ml. To our surprise, the volume of the medium turned out to be a crucial limiting factor for swarming motility. Bacteria are not capable of swarming on plates containing more than 10ml of medium, with the optimal volume for swarming being 5ml (Fig. 12). Consequently, swarming assays of *D. solani* cannot be performed on plates containing the canonical 25ml of medium. For technical reasons related to the rapid solidification of the agar and the need for a smooth surface, we decided to use plates containing 7.5 ml of medium as standard for our motility assays.

It is well documented that swarming motility in Gram-negative plant pathogens is under control of quorum sensing (QS) (Köhler et al., 2000). Two synergistic quorum sensing mechanisms have been identified in *Dickeya* species: the Exp system based on classic N-acyl-homoserine lactone (AHLs) signals and a specific system depending on the production and perception of a molecule of unknown structure, Virulence Factor Modulating (VFM). While the VFM sensing system seems to regulate the production of cell-wall degrading enzymes, the AHLs system has proved to be involved in swarming and swimming motility of other *D. solani* strains. (Potrykus, et al. 2018).

To establish effective social movement, bacteria need to produce and sense the presence of the AHLs, small diffusible molecules, that act as autoinducers, resulting in a positive feedback loop that activates QS and the motility genes. The quorum sensing mechanism has been intensively studied in bacteria: the autoinducer needs to accumulate to sufficiently high concentrations in the cell proximity to reach the threshold of activation and trigger the bacteria decision of synchronize behaviour. Consequently, the autoinducer has two roles: to informing bacteria that the minimum cell-density has been reached and to activate the social behaviour. However, the secretion of the autoinducer is not the only player in the sensing-mechanism. As diffusible molecules, the AHLs can diffuse in the medium, making bacteria not capable of responding to the cell density. In fact, iIt has been intensively shown that cells within biofilm accumulate high level of AHLs and undergo QS induction sooner than cells at the periphery of the biofilm as AHLs can be dissipated by the diffusional losses. (De Kievit et al., 2001).

Understanding swarming motility needs to account for the effects of the agar on the diffusion of the signalling molecules. Whether the signal is capable of reaching the

activation threshold depends on the cell density but also on the balance between loss and retention of the AHLs in the proximity of the colony. (Trovato et al., 2014). Therefore, when QS-based social behaviours are induced in laboratory conditions, the diffusion rate of the autoinducer in the artificial media cannot be ignored.

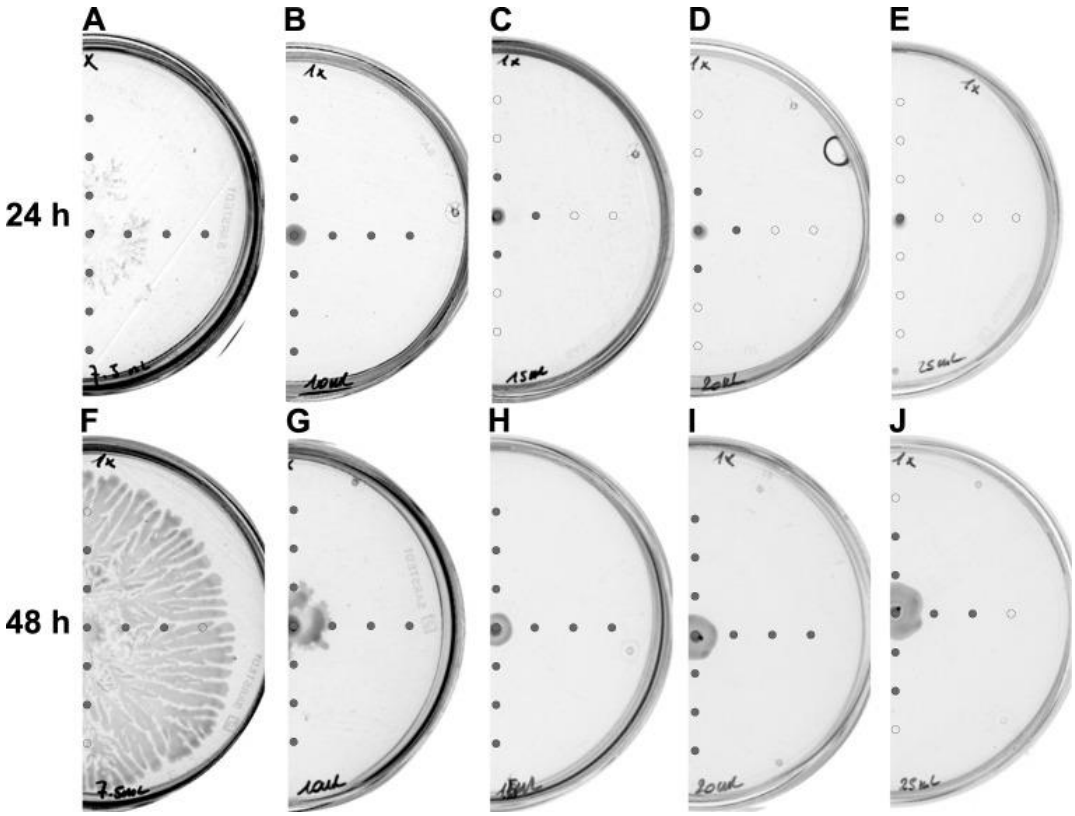

Figure 12 -Swarming and AHLs production by Dickeya solani IFB102 on plates containing following volumes of B-medium: (A, F) 7.5 ml, (B, G) 10 ml, (C, H) 15 ml, (D, I) 20 ml, (E, J) 25 ml. Swarming assays were performed for 24 and 48 h, as indicated. Grey circles represent spots at which AHLs were detected. Open circles represent spots at which no AHLs were detected.

We wanted to verify whether the decision to swarm or not to swarm, correlates with the radial diffusion of AHLs in the medium. After incubation, the medium was sampled and tested for presence of AHLs using the biosensor strain *E. coli* PSB401 (Winson et al., 1998). After 24h, AHLs were detected at all tested spots of plates containing 7.5 ml and 10 ml of medium. On the plates where swarming did not occur, the detection of AHLs was either limited to the area of the colony growth (Fig. 12 E) or limited within 1 cm radius from the point of inoculation (Fig. 12 C and D). Prolongation of the incubation time up to 48 hours changed the pattern of AHLs distribution in the medium. In the plates containing 7.5 ml of medium, where we observed the most efficient swarming of *Dickeya*, the radius of AHLs detection was reduced down to 2 cm from the inoculation spot (Fig. 12 F). In the plates with 10, 15, and 20 ml of the medium we detected AHLs at every

tested spot (Fig. 12 G–I). In the case of the plate containing 25 ml of medium AHLs were detected within 2 cm radius from the inoculation spot (Fig. 12 J).

In the natural environment, bacteria swarm on plant leaf to reach their target. The leaf represents a relatively hydrophobic environment limiting the rapid diffusion of the AHLs through the cuticle (Bucholz et al., 1998). A thin layer of agar is a closer representation of the natural environment and allows a faster radial diffusion of the signalling molecules limiting the dispersion of the AHLs in the depth of the agar. In fact, we could observe an increase in the area of the plate with detectable AHLs as the volume of the medium decreased, allowing cells to sense the AHLs threshold earlier, as the molecules are confined in a smaller space.

7.3 Influence of medium concentration on swarming motility and quorum sensing

When it comes to energy consumption, swarming motility is extremely expensive as it requires the synthesis of a large number of flagella and occurs at a high growth rate (Kearns et al., 2010). Consequently, swarming is never a process that takes place under starvation and requires access to a source of nutrients. Some bacteria as *Bacillus subtilis* (Julkowska et al., 2005), *Salmonella typhimurium* (Berg et al., 2005), *Rhizobium leguminosarum* (D. Tambalo et al., 2010) *Proteus mirabilis* (Wilkerson et al., 1995) are known to swarm on a variety of energy-rich media, while others such as *Salmonella enterica* (Kim et al., 2005) have been shown to require specific supplements like glucose in order to exhibit swarming behaviour.

We have shown that *D. solani* prefers minimal medium to exhibit swarming motility which, on the other hand, is very limited on rich media (Fig. 9). To assess how the availability of nutrients can impact the swarming of *D. solani* we performed swarming assay on synthetic B-medium while increasing the content of its single components.

On media containing 7.5ml o 0.5x and 1x concentrated B-medium components, swarming motility occurs in 24h (Fig. 13). Increasing the medium concentration to 1.5x results in a delay of swarming which is visible only after 48h. At the highest tested concentration (Fig. 13 D-E), *D. solani* exhibited no swarming behaviour at 24h with the growth restricted to a central colony. However, when the incubation time was extended to 48 hours (Fig. 13 I and J), the growth on the plate became more visible. This suggests that the high concentration of components could slow down cell division and population

growth, thereby limiting the ability to produce effective movement. Prolongation of the incubation time did not trigger swarming motility (data not shown). It is likely that the longer incubation time led to the evaporation of the water content in the plate and to a less wet surface, which is not suitable for swarming motility. Therefore, the maximum incubation time for the swarming assay was fixed at 48 hours.

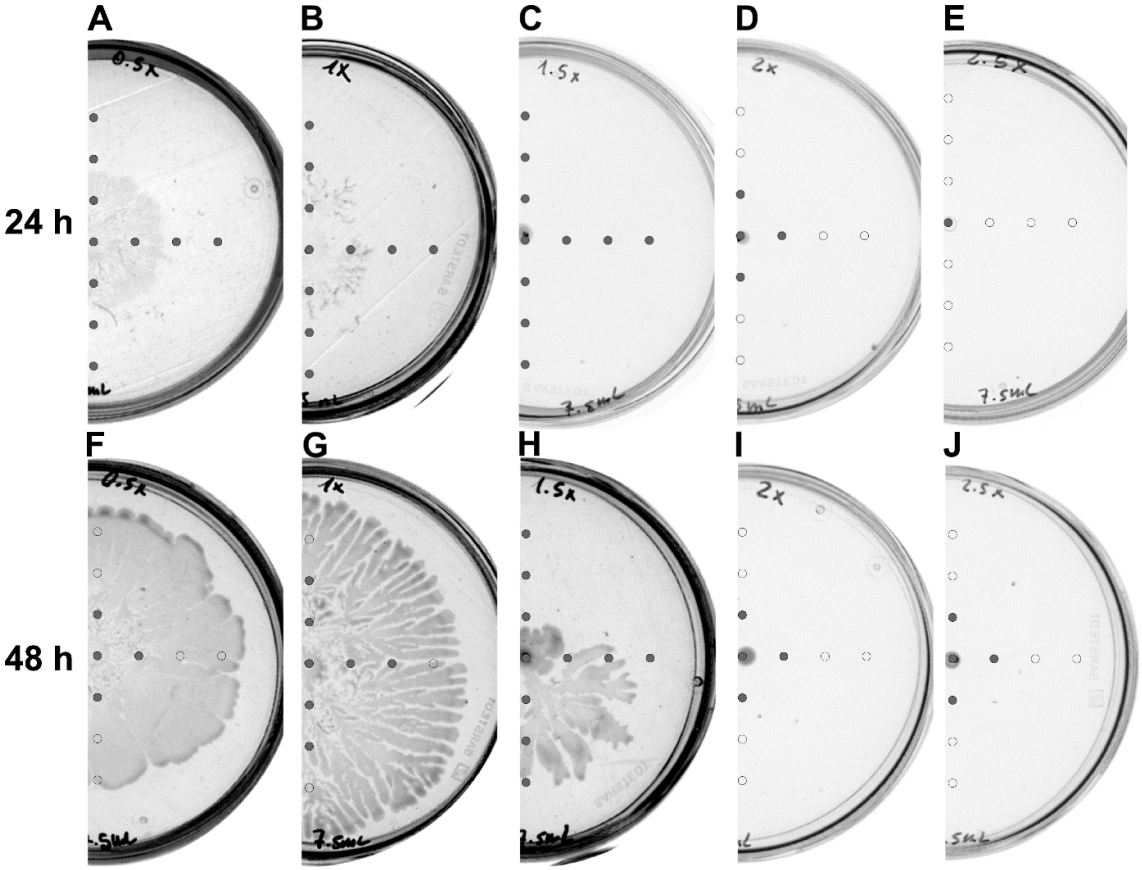

Figure 13 – Swarming and AHLs production by Dickeya solani IFB102 on plates containing 0.5% of agar and the changing concentrations of B-medium: (A, F) 0.5x, (B, G) 1x, (C, H) 1.5x, (D, I) 2x, (E, J) 2.5x. Swarming assays were performed for 24 and 48 h, as indicated. Grey circles represent spots at which AHLs were detected. Open circles represent spots at which no AHLs were detected.

The pattern of AHLs distribution in the medium changed along with the increase of medium concentration and incubation time. For 24 hours incubation we detected AHL molecules all over the plates containing 0.5x, 1x, and 1.5x medium (Fig. 13 A–C). In the case of plates with 2x concentrated medium, we could detect AHLs within 1 cm radius from the inoculation spot (Fig. 13 D). Sampling of plates containing 2.5x concentrated medium allowed us to detect AHLs only at the inoculation spot (Fig. 13 E). The 48-hour incubation changed the distribution of AHLs in the tested plates. In the plates containing the least concentrated medium (0.5x), we detected AHL molecules within 1 cm radius from the inoculation spot (Fig. 13 F). The radius of AHLs detection increased up to 2 cm in the case of plates with 1x medium (Fig. 13 G) and up to at least 3 cm from the

inoculation spot as observed for the plates containing 1.5x medium (Fig. 13 H). Further increase in medium concentration (2x, 2.5x) resulted in decreasing the radius of AHLs detection down to 1 cm from the inoculation spot (Fig. 13 I and J).

We wanted to verify whether the swarming/no swarming pattern that we observed while changing the medium concentration could also be affected by the volume of the medium. Thus, we repeated the media-concentrated swarming assay on plates containing an increasing volume of medium. Our results confirmed that the increasing the volume of the medium affects the diffusion of the AHLs and slows down motility at all tested concentrations (Tab. 2).

Medium Concentration	Glucose Concentration	Volume of Medium					
		7.5mL	10mL	15mL	20mL	25mL	
0.5x	0.25%	++++	+++	+	-	-	Swarming
		++	++	++++	++++	+++	AHLs
1x	0.5%	++++	++	+	+	+	Swarming
		++++	++++	++++	++++	+++	AHLs
1.5x	0.75%	++	+	-	-	-	Swarming
		++++	+++	+++	+++	++	AHLs
2x	1%	-	-	-	-	-	Swarming
		++	++	++	++	+	AHLs
2.5x	1.25%	-	-	-	-	-	Swarming
		++	++	+	+	+	AHLs

Table 2 - Summary of swarming and AHLs production of D. solani IFB102 in B-medium (0.5% of agar) in plates containing different volume of medium and containing medium differently concentrated. "+" indicates the presence of swarming/detection of AHLs at (+) center; (++) 1 cm; (+++) 2 cm; (++++) 3 cm from the point of inoculation 24h upon incubation.

Our results confirm that the diffusion of AHLs in the medium plays a crucial role in triggering swarming behaviour and that swarming of *Dickeya solani* is also affected by the media composition; some nutrients are required to sustain bacteria growth and proliferation, but an excess of nutrients can slow down proliferation and have a negative impact on motility.

7.4 Influence of glucose on swarming motility

The synthetic B-medium contains amino acids and glucose to promote bacteria proliferation and growth. While amino acids are known to support motility, glucose has been shown to affect the synthesis of flagella and reduce motility in other Enterobacteriaceae (Adler et al.,1967). While increasing the content of the medium in the previous experiment, we also increased the concentration of glucose in the medium (from

0.25%, contained in the 0.5x concentrated B-medium, to 1.25% contained in the 2.5x concentrated medium). Therefore, we wanted to verify whether the amount of glucose, which is the only sugar contained in the B-medium, could influence the ability of *D. solani* to produce effective movement on plates.

In order to gain deeper insights into the impact of glucose on swarming motility, we conducted swarming assays by varying the glucose concentration in a 0.5x concentrated B-medium, which we determined to be the optimal composition for promoting swarming (as indicated in the Tab. 2). Following 24-hour incubation from the time of inoculation, distinct swarming patterns of *Dickeya* were observed (Fig. 14). These patterns can be categorized into four groups: (i) a small central colony without visible swarming (0% to 0.1% of glucose, Fig A–D), (ii) a central colony with the increasing ring of swarming bacteria (0.25% to 0.4% of glucose, Fig. 14 E–G), (iii) a small colony at the inoculation spot with extending irregular dendrites (0.5% to 3% of glucose, Fig. 14 H–M), (iv) a large uniform central colony without dendrites (4% and 5% of glucose, Fig. 14 N and O).

The distribution of AHL molecules in the medium was found to be dependent on the concentration of glucose. When the glucose content was at its lowest levels (0%, 0.01%, and 0.02%), the radius within which AHLs were detected extended up to 2 cm from the point of inoculation (Figures 4A–4C). However, in plates containing glucose concentrations ranging from 0.1% to 1%, AHLs were detected within a radius of at least 3 cm from the inoculation spot (Figures 4D–4I). For plates with glucose concentrations between 1.5% and 3%, AHL molecules were primarily detected in the region of the medium that was covered with swarming bacteria (Figures 4J–4M). Remarkably, when the glucose content reached its highest levels (4% and 5%), no AHL molecules were detectable at any of the tested spots (Fig. 14 N and 4O).

Obtained results suggest that glucose affects motility and swarming pattern: some minimal concentration of glucose (at least 0.25%) is required to trigger *Dickeya* swarming motility; high glucose content (4% and 5%) may hinder or suppress the formation of dendritic extensions and potentially impact the overall swarming ability of the bacteria.

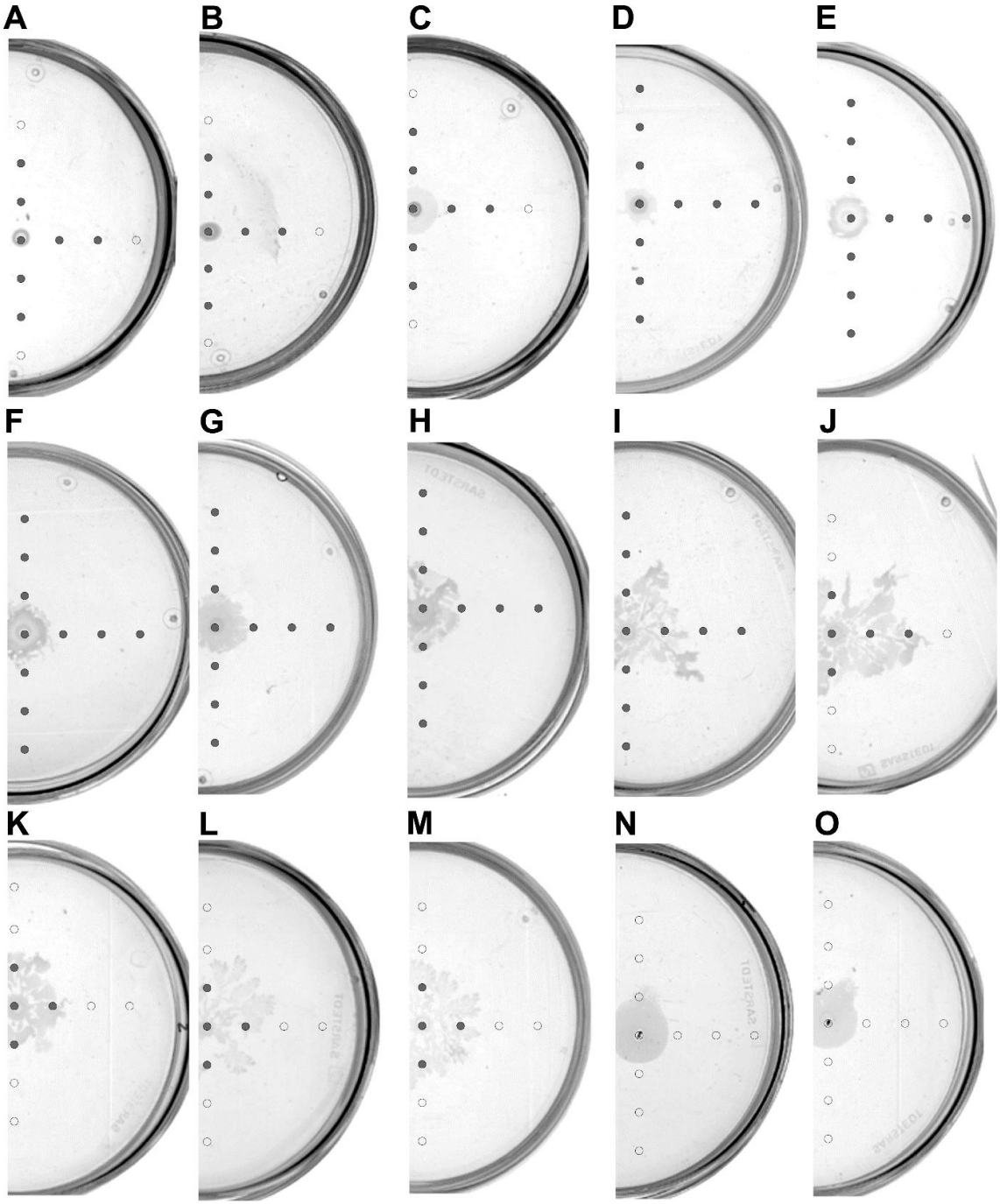

Figure 14 - Swarming and AHLs production by Dickeya solani IFB102 on plates with B-medium containing 0.5% of agar and the following concentrations glucose: (A) 0%, (B) 0.01%, (C) 0.02%, (D) 0.1%, (E) 0.25%, (F) 0.3%, (G) 0.4%, (H) 0.5%, (I) 1%, (J) 1.5%, (K) 2%, (L) 2.5%, (M) 3%, (N) 4%, (O) 5%. Swarming assays were performed for 24 h. Grey circles represent spots at which AHLs were detected. Open circles represent spots at which no AHLs were detected

As high levels of glucose proved to be a limiting factor for swarming motility, we wanted to exclude the influence of high level of glucose on the inhibition of swarming registered in concentrated B-medium. Therefore, we repeated the motility assay on plates containing concentrated medium in the range from 0.5x to 2.5x, while keeping the medium volume at 7.5ml and concentration of glucose at 0.5%, as it resulted in the optimum content from our previous experiment (Tab. 3)

Medium Concentration	Glucose Concentration	Volume of Medium						
		7.5mL	10mL	15mL	20mL	25mL		
0.5x	0.5%	++	++	+	-	-	Swarming	
		++++	++++	+	-/+	-/+		48h
		++++	++++	++	+	+	AHLs	
		+	+	++++	+++	+++		48h
1x	0.5%	++	-	-	-	-	Swarming	
		++++	++	+	+	+		48h
		++++	++	++	++	+	AHLs	
		++++	++++	++++	++++	+++		48h
1.5x	0.5%	-	-	-	-	-	Swarming	
		+	+	+	+	+		48h
		++++	++	++	++	++	AHLs	
		++++	++++	++++	++++	++++		48h
2x	0.5%	-	-	-	-	-	Swarming	
		-	-	-	-	-		48h
		++	++	++	++	++	AHLs	
		++	++	++	++	++		48h
2.5x	0.5%	-	-	-	-	-	Swarming	
		-	-	-	-	-		48h
		++	++	++	++	++	AHLs	
		+	++	++	++	++		48h

Table 3 – Swarming and AHLs production of D. solani IFB102 on medium containing 0.5% of glucose and increasing concentration of the other B-medium components: 0.5x, 1x, 1.5x, 2x, 2.5x; on plates containing an increasing volume of medium: 7.5mL; 10mL; 15mL; 20mL; 25mL. Plates were incubated at 28°C for 48h. "-" indicates no swarming or AHLs detected; "+" indicates the degree of detection of swarming or AHLs.

At 48h upon incubation (Tab. 3), the swarming pattern repeated what we observed in the previous experiment: a delay in swarming on plates with 1.5x concentrated medium, and complete inhibition of swarming in all samples with 2x and 2.5x concentrated medium, despite the lower glucose content of 0.5%. These findings indicate that the inhibition of *Dickeya* swarming is not solely dependent on increasing glucose levels. It suggests that other components present in the B-medium also play a significant role in influencing the swarming behaviour of *Dickeya*. Thus, the inhibitory effect on swarming is likely a result of a combination of factors rather than solely attributable to glucose concentration.

The influence of glucose on swarming motility of *Dickeya solani* was not unexpected. Swarming relies on the flagella synthesis and previous studies have established that glucose can prevent synthesis of flagella in many bacteria species. For instance, Shrout

et al (2020) have investigated the effect of glucose on *Pseudomonas aeruginosa* and reported that its presence leads to compromised surface motility and altered biofilm structures, resulting in limited swarming motility and the formation of cellular-aggregate biofilms. Similarly, Jahid et al. (2013) demonstrated that the addition of glucose influences swarming motility and quorum sensing of *Aeromonas hydrophila*, a foodborne pathogen, with complete inhibition of swarming observed upon the addition of 1.0% and 2.5% glucose. Furthermore, the relationship between glucose and swarming motility extends to *Proteus mirabilis*, a pathogenic enterobacterium extensively studied as model for swarming motility. Armitage et al. (1981) have shown a direct link between swarming in *Proteus mirabilis* and the cellular level of cyclic adenosine monophosphate (cAMP). Specifically, the presence of 1% glucose during growth has been shown to cause a decline in cAMP levels, resulting in the inhibition of swarming.

Our observations on the inhibitory effect of glucose on swarming motility of *Dickeya solani* aligns with these findings, as glucose likely interferes with flagella synthesis, impairing the assembly and function of the flagella-driven swarming phenotype. To date, the only known regulatory mechanism for glucose in swarming involves the cAMP-dependent transcriptional regulation of flagellar genes (Park et al., 2016). This regulatory process can be attributed to carbon catabolite repression (CCR), which allows bacteria to prioritize glucose utilization over other carbon sources to support efficient growth (Gorke and Stulke, 2008).

In *E. coli*, the enzyme adenylate cyclase (cyaA) generates cAMP in response to environmental glucose levels. The cAMP-receptor protein CRP, encoded by the *crp* gene, acts as a transcriptional factor that binds to cAMP, modulating the expression of multiple genes involved in motility, including those related to flagella synthesis such as *flhDC*. The prevailing model suggests that increased cAMP levels, resulting from less efficiently metabolized carbon sources, promotes flagellum synthesis, while conditions that decrease cAMP levels, such as high glucose, inhibit the synthesis to adapt to glucose-rich environments. Consequently, bacteria exhibit reduced motility in high glucose environments and enhanced motility in environments with less efficient carbon sources. (Stella et al., 2008).

In other *Dickeya* species, CCR also plays a regulatory role in the expression of major pectinase genes involved in virulence, with the direct implication of the cAMP receptor

protein (CRP). This evidence suggests a possible link between CCR and quorum sensing (Reverchon et al.,1997). Moreover, in *Pseudomonas aeruginosa*, gene expression is regulated by two interconnected quorum sensing systems: AHL-based (las and rhl) and non-AHL-based (qps and iqs), which respond to changes in bacterial population density. These quorum sensing systems have been shown to significantly influence the virulence of *Pseudomonas aeruginosa*. Furthermore, these systems are associated with carbon catabolite repression (CCR), facilitated by protein quality control (PQC) proteases Lon and ClpXP. Under control of CCR, the AHL-based quorum sensing systems las and rhl specifically regulate the production of rhamnolipids, crucial biosurfactants for promoting efficient swarming motility in *Pseudomonas aeruginosa*.

Accordingly, in addition to the glucose-dependent changes in swarming patterns, we also observed significant variations in the distribution of AHL quorum sensing molecules in the medium. This finding indicates a tight association between glucose concentration, swarming behaviour, and quorum sensing in *Dickeya solani*. Although a direct link between these factors has not been established so far, a study by Potrykus et al. shed light on the influence of quorum sensing systems in *Dickeya solani* on critical biological processes such as plant tissue maceration, production of plant cell wall-degrading enzymes, and swarming motility. To the best of our knowledge, this study is the first to show a clear indication of a close association between glucose concentration in the medium, swarming behaviour, and quorum sensing (Gatta et al., 2022).

8 *Bacillus subtilis* MB73/2 and *Dickeya solani* IFB102 swarming and social interaction

Plant surfaces represent intricate ecosystems hosting diverse microbiota where multiple species coexist as dynamic communities. The root surface and surrounding rhizosphere are significant carbon sinks, primarily generated by the plant itself (Compant et al., 2005). As a result, nutrient-rich niches generate along the root surfaces, attracting a wide range of microorganisms, including phytopathogens and biocontrol bacteria (Nelson et al., 2004). In this highly competitive ecosystem, the ability of cells to act as a team and swarm proficiently is a competitive advantage that has provided significant benefits to bacteria in host colonization, formation of biofilms, defence against competitors, and adaptation to changing conditions (Lì and Tian., 2012). Co-existing species utilize a wide range of strategies to effectively warfare their neighbours or interact with cooperating bacteria, secreting molecules (e.g., enzymes, surfactants, matrix components) which act as repellents or as public goods for the communities.

B. subtilis MB73/2 and *Dickeya solani* IFB102 were selected as putative antagonistic species (Preliminary Results). Both species naturally inhabit the plant rhizosphere, prompting us to explore whether they employ competitive strategies to either indirectly compete for resources or actively interfere with each other through the release of antimicrobial compounds.

Research on bacteria multi-species interactions typically involves co-inoculating bacteria in liquid media and cultivating them planktonically. This allows to determine synergy in growth rate, carrying capacity, or total live colony-forming units (CFUs). Under these experimental settings, an increased growth rate of both partners is interpreted as mutualism, while a decreased abundance of both implies competition. Then, in case of competition, the antagonistic species is cultivated in a concentrated crude extract from the opponent (CFS). This approach has been useful in determining minimal inhibitory concentrations and in providing evidence for whether an effector molecule is secreted at concentrations that are bioactive against the competitor. However, planktonic cultures often do not represent natural systems. Thus, we combined a classic approach to the interaction with an alternative method of screening social interaction on semi-solid agar, where interacting molecules can diffuse through the agar at concentrations that mimic natural systems more closely. Many bacterial species activate transcription of biofilm-

related genes on solid media and differentiate into spatial heterogeneity, increasing the accuracy (and complexity) of the interaction system. Therefore, to investigate the social dynamics at play, we performed a series of experiments to analyse how the interacting populations respond to the presence of their competitor when they establish social interactions (co-culture on a semi-solid medium) and when they act as single free-living cells (liquid co-culture).

8.1 *Bacillus subtilis* MB73/2 and *Dickeya solani* IFB102 antagonistic interaction on synthetic semi-solid B-medium

We conducted a comprehensive investigation into the potential antagonistic interaction between *Bacillus subtilis* and *Dickeya solani* when they interact as cell collectives. To do this, we performed swarming motility assay under laboratory conditions that were previously characterized as suitable for swarming of both species.

Equal amounts (2 µl of refreshed culture at OD600=0.2) of bacteria were inoculated in a Petri plate containing 0.5x concentrated B-medium supplemented with 0.5% of agar. Bacteria were inoculated at a distance of 1.5 cm, providing adequate space for independent growth while allowing interactions.

After an incubation of 24 hours, we could macroscopically register an architecturally complex phenotype. *B. subtilis* created a highly wrinkled swarm pattern with a defined center and dense dendrites spreading from the central colony in all directions. Interestingly, the swarming of *B. subtilis* was interrupted at ~ 0.3 cm from the front of inoculation of *D. solani*. Additionally, when the two colonies came into proximity, *D. solani* swarmed in the opposite direction. Within a few hours, the central colony of *Dickeya solani* was translocated entirely from the point of inoculation to the edge of the plate, moved by a distance of ~ 2.5 cm.

Zooming in on the point of inoculation, we could see that the area of inoculation was inhabited by *D. solani* cells with a much lower density compared to the edge of the dendrites. In addition, cells at the inoculation point did not display the elongate swarm morphology (Fig. 15 C).

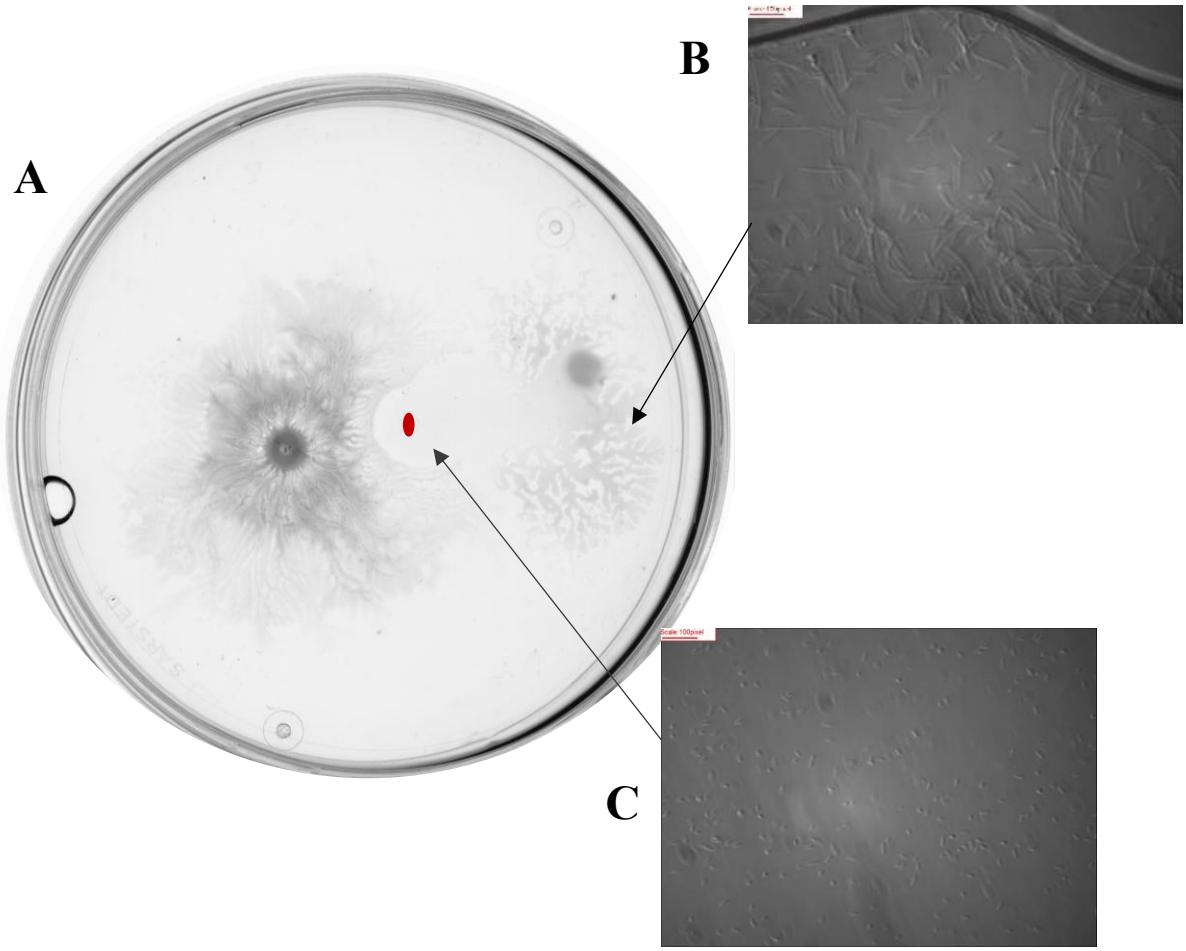

Figure 15 - (A) B. subtilis MB73/2 and D. solani IFB102 *social interaction on a swarming plate containing 0.5x B. medium with 0.5% of agar. Bacteria were inoculated at a distance of 1.5 cm and plate were observed 24h after inoculation. Red dot indicates the inoculation point of D. solani. (B) Example of magnification of inoculation zone under optical microscope. (C) Example of magnification of D. solani tendrils under optical microscope.*

Therefore, it is highly likely that the point of inoculation remained inhabited by a small population of cells, within *Dickeya solani* population, that were unable to activate swarming motility. Considering the distance covered, it is evident that the observed bacterial movement is driven by active propulsion, meaning that bacteria are actively moving in a directional and coordinated manner to escape from the antagonistic *B. subtilis*.

To better understand the interaction, we limited the incubation to 6 hours, time required to pass the lag phase and initiate the growth. In order to optimize data collection, we minimized the distance between the interacting species to 0.5 cm. Unfortunately, obtaining a complete time lapse proved challenging due to the specific incubation requirements for *D. solani* to establish swarming behaviour: removing the plate from the incubator resulted in variations in experimental conditions and halted the swarming process. Despite these limitations, the data collected during the observed period provided

valuable insights into the initial stages of the bacterial interaction. It is worth noting that *D. solani* swarming is slightly delayed, occurring approximately 1 hour later than *B. subtilis*. As a result, at this reduced distance, MB73/2 approaches IFB102 precisely when the colony has reached the cell density necessary for swarming motility but has not yet entered the actual swarming phase. (Fig. 16) Therefore, at the time when the swarming *B. subtilis* approached, *D. solani* growth was still confined to the point of the inoculation. Even so, it is clearly visible an increased cell density in the area of the colony diametral opposite to MB73/2, suggesting that the growth rate is already affected by the close presence of *B. subtilis*. Moreover, when bacteria entered into swarming motility, the formation of dendrites was, from the real beginning, limited to the area opposite to MB73/2, suggesting that *D. solani* is not even attempting to swarm in the direction of *B. subtilis*.

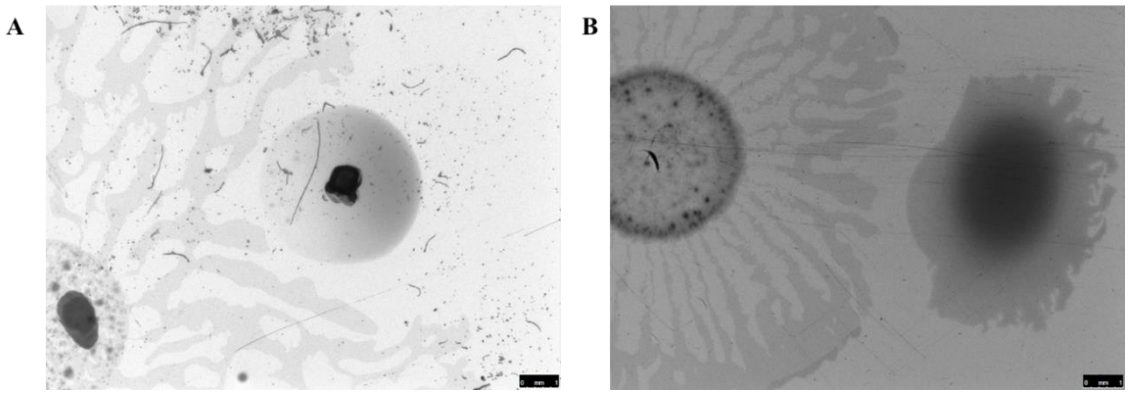

Figure 16-B. subtilis MB73/2 and D. solani IFB102 *social interaction on a swarming plate containing 0.5x B. medium with 0.5% of agar. Bacteria were inoculated at a distance of 0.5 cm and plate were observed 6h after inoculation. (A) Swarming of* IFB102 *is not yet visible and bacteria are still growing. (B) Swarming lag is passed and* IFB102 *forms tendrils in the opposite direction to MB73/2.*

This interaction has been identified as a standard or "wild-type phenotype" and has served as a baseline for subsequent experiments. Therefore, this assay has been repeated several times, in different seasons and environmental conditions. Factors such as the experimental setup significantly influence the size of the inhibition zone and the extent of translocation. For example, the length of *B. subtilis* lag phase can affect how quickly these bacteria encounter *D. solani*. If this encounter occurs before *D. solani* reaches a specific population density, the resulting inhibition zone will be smaller (Fig. 17), and *D. solani* escape response will be more rapid. For this reason, we decided not to consider the diameter of the inhibition zone as a quantitative measure, but rather qualitative.

It is worth considering that swarming, defined as the coordinated and directional movement of a group as a whole, is not limited to the world of bacteria. It is a prevalent phenomenon found across various levels of living organisms and widely observed in nature, such as in fish shoals, flocks of birds and herds of mammals (Shklarsh et al., 2011). Swarming serves as a crucial predator avoidance mechanism, at all levels, enabling effective escape from potential threats. Common features of swarm-predator interactions are (i) the formation of an empty space surrounding the predator and (ii) the presence of a relatively sharp boundary of the swarm, indicating the swarming group's concerted effort to maintain distance from the threat. (Chen and Kolokolnikov, 2014). The observed phenotype resulting from the interaction between *D. solani* and *B. subtilis* closely mirrors the predator-avoidance mechanisms widely observed in nature. However, while in the realm of macro-organisms, it's relatively straightforward to comprehend how prey can detect the presence of a predator, in the bacterial world, communication takes place at the molecular level and requires a deep understanding of gene regulation and induction which controls what bacteria can secrete and detect.

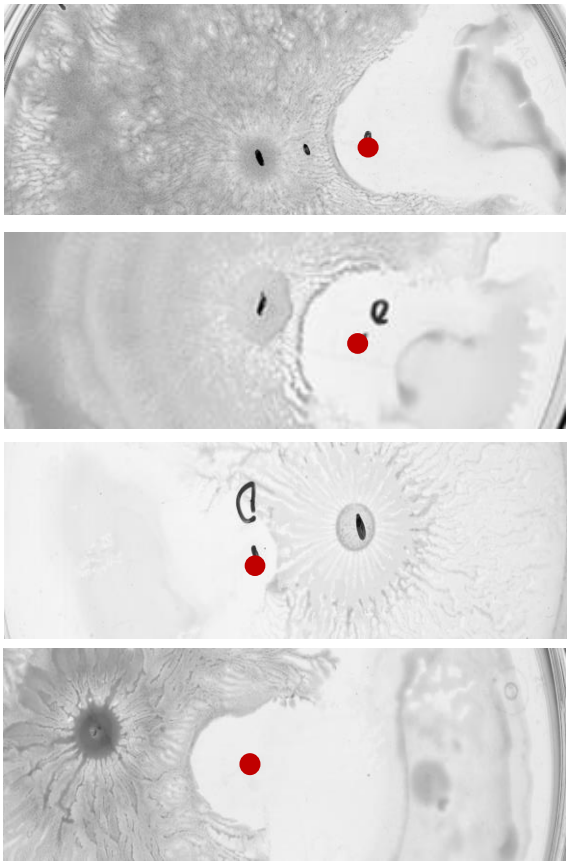

Figure 17- Replicates of B. subtilis MB73/2 and D. solani IFB102 *swarming interaction on synthetic B. medium. Red dots indicate the inoculation point of D. solani*

Over the past half-century, various degree of cross-talk or cross-inhibition have been observed among bacteria and our understanding of the molecular mechanisms, signal structures, gene regulons, and behavioural responses linked to bacterial social interactions has grown significantly. Nonetheless, many studies focused on the altered behaviours and production of secondary metabolites in homogenous systems with single species cultures, due to the complexity of the experimental settings involving multi-species interactions.

Some bacteria, including *P. dendritiformis* (Be'er et al., 2009) and *Bacillus subtilis* (James et al., 2009) exhibit a form of sibling-avoidance. Sibling bacterial colonies of *P.*

dendritiformis, secrete an unidentified inhibitory compound termed sibling lethal factor (Slf) which serves to control the colony growth in energy-limited environments. Similarly, *B. subtilis* strains examined on swarm plates in pairwise combinations were found to distinguish phylogenetically unrelated form phylogenetically related strains, forming either distinct boundaries (non-kin strains) or the swarms merge (kin strains). Interestingly, the non-kin bacteria competed with each other and only one was able to colonize plant roots. In fact, the swarm fronts emerging from two locations in the same swarm plate led to the formation of a margin line of inhibition unpopulated by either of the advancing swarm fronts which was viewed by the authors as a form of self-avoidance, an antagonistic mechanism preventing the coexistence of non-kin *B. subtilis* on roots (Stefanic et al., 2015). However, the registered interaction between *B. subtilis* and *D. solani* is not limited to the formation of an avoidance line. Instead, we identified the formation of an inhibition zone that *B. subtilis* is not capable of penetrating and *D. solani* avoids colonizing, escaping in the opposite direction. This active movement made us hypothesize that the inhibition zone represents a more complex interaction zone where both species secrete molecules with antagonistic properties. *B. subtilis* secretes an antimicrobial compound that repels *D. solani* and induces a change in the swarming direction, while *D. solani* produces an antimicrobial compound that prevents *B. subtilis* from approaching.

In fact, at the front of the MB73/2 interaction zone, there is a clear and noticeable increase in cell density, which promotes the formation of a multi-layered wrinkled biofilm, distinct from the biofilm thickness of the dendrites observed at the edge of the plate (Fig. 18 and Fig. 19). These findings align with the theory proposed by Grobas et al., 2021, where the authors demonstrated that a stress-response mechanism at the collective level could trigger biofilm formation through a mechanochemical feedback loop. The presence of kanamycin was able to trigger the synthesis of extracellular matrix and induce biofilm formation in a swarming colony front of *B. subtilis* on soft agar, in a concentration-dependent manner. Similarly, the presence of *D. solani* is perceived by *B. subtilis* as a potential threat, leading to the initiation of stressor-triggered biofilm formation, which occurs in conjunction with the release of repellent compounds.

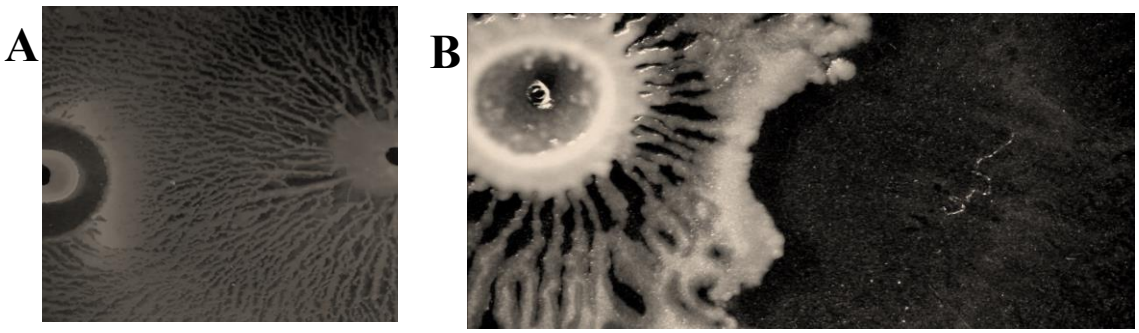

Figure 18 - Formation of a multi-layered wrinkled biofilm of B. subtilis at the edge of the inhibition zone upon interaction with D. solani IFB102 *in conditions that allow swamring of D. solani (B) and in conditions that do not support swarming of D. solani*

Antagonistic interactions resembling prey-predator dynamics are not uncommon in the bacterial world, with many bacterial species exhibiting some degree of predatory behaviour (Vasse et al., 2024). Myxobacteria, for example, are renowned as generalist predators that produce enzymes like proteases, lysozyme, amidases, and endopeptidases upon encountering a suitable prey. This predatory strategy involves social interactions, with individual cells aggregating to form fruiting bodies and collectively swarming through prey colonies in ripple-like waves (Kaimer et al., 2023). Myxobacteria can prey both Gram-positive and Gram-negative bacteria. However, even in the presence of such aggressive behaviour, the "prey" does not remain passive. For instance, *B. subtilis* environmental strains effectively produce bacillaene that confers resistance to *M. xanthus* predation (Muller et al., 2014).

Production of secondary metabolites in response to the presence of an antagonistic species has been documented in many bacteria. For instance, a number of studies have shown that presence of *B. subtilis* soil isolates can induce production of secondary metabolites in *S. coelicolor* that are not produced when the bacterium is grown as a single culture (Traxler et al., 2014). Similarly, *P. aeruginosa* demonstrates a sophisticated adaptive response upon detection of diffusible signaling factor (DSF) produced by *Stenotrophomonas maltophilia*. This recognition prompts *P. aeruginosa* to augment its defense mechanisms by synthesizing increased quantities of exopolysaccharides and inducing membrane modifications (Ryan et al., 2008).

In the interaction between MB73/2 and IFB102 the dynamic at play is much more complex than an avoidance interaction as it necessitates both bacteria to sense the presence of each other and respond to the perceived threat. The mutual secretion of antagonistic compounds adds another layer of complexity to the interaction, further highlighting the sophisticated strategies employed by bacteria in response to each other's

presence. A recent model introduced by Barua and collaborators advanced the hypothesis that cells can be seen as decision-making machines that can sense changes in the surrounding microenvironment and respond accordingly (Barua et al., 2023). However, cells constantly receive numerous signals that are integrated by the intrinsic signal transduction system and must distinguish between noisy and important signals. For instance, producing an antimicrobial compound and changing the direction of motility is extremely expensive in terms of energy consumption, thus, cells must carefully assess the significance of the perceived threat before committing to such resource-intensive actions.

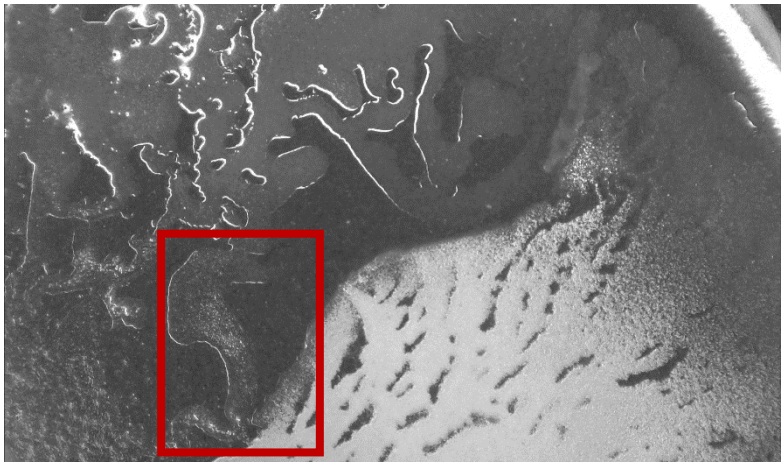

Figure 19- Confluent swarming fronts of D. solani IFB102 *(up) and B. subtilis MB73/2 (down) at the edge of a swarming-assay Petri plate.*

It has been suggested that the production of antimicrobial compounds hinges on three pivotal factors: a high density of self-cells, a correspondingly high density of other cells, and a later growth stage. A high density of self-cells implies that the toxin will be released in considerable amounts and will be effective. A high density of other cells means that many target bacteria are present that might otherwise represent a threat. And, at a late growth stage, the most essential early growth phase is over and the costs of investing in toxin secretion are relatively low. This theory aligns with our fundings, where *B. subtilis* cells at the late stage of development (inoculation point) exhibit stronger repellent propriety and a thicker biofilm at the point of interaction compared with the edges of the dendrites where the two swarmer's fronts (*B. subtilis* and *D. solani*) can indeed merge (Fig. 19).

8.2 *B. subtilis* MB73/2 triggers motility of *D. solani* IFB102 under conditions that prevent its swarming

Our findings suggest that the swarming behaviour of *D. solani* is highly contingent upon specific environmental conditions (Gatta et al., 2022). Leveraging this understanding, it is possible to selectively inhibit the swarming of *D. solani* while simultaneously fostering swarming of *B. subtilis*. Therefore, to better understand the intricate antagonism we performed swarming motility assay on plates containing 25ml of 0.5x B-medium with 0.5% agar and on plates containing 7.5ml of 0.5x B-medium with 0.7% of agar. The medium volume and the agar concentration are both impairing the swarming motility of *D. solani* limiting the diffusion of quorum sensing molecules or the motility itself (Gatta et al., 2022).

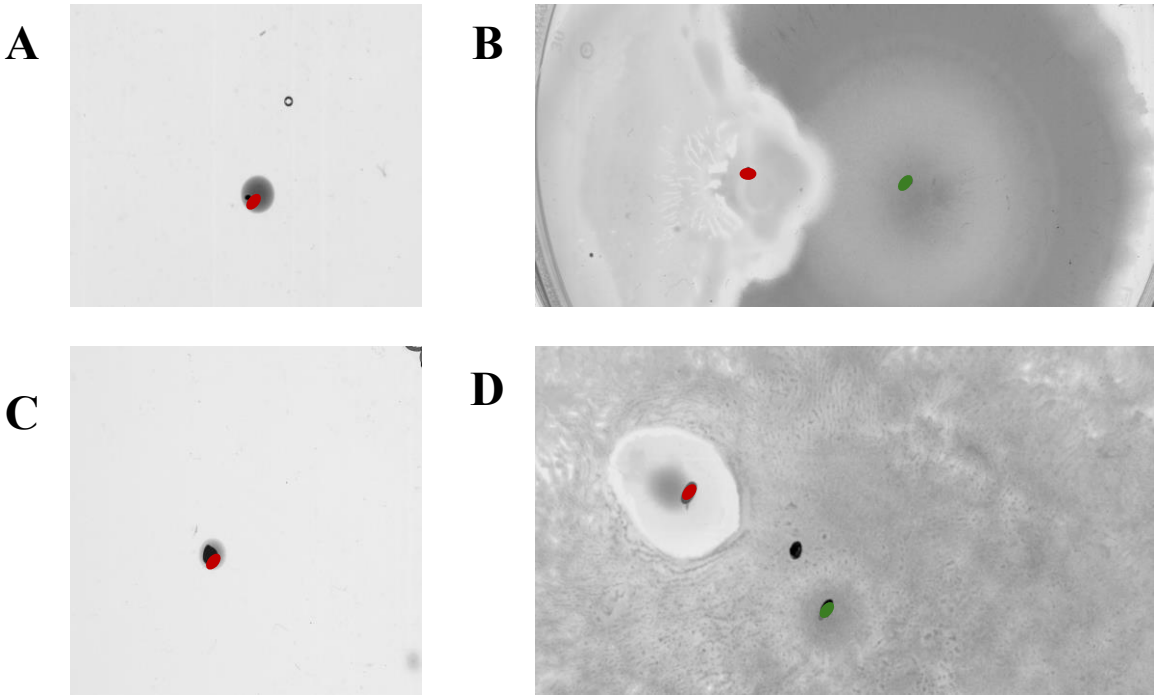

*Figure 20 - Swarming assay of D. solani IFB102 on (A) 25*ml *of 0.5x B-medium with 0.5% of agar and on (C) 7.5*ml *of 0.5x B-medium with 0.7% of agar. Swarming interaction of B. subtilis MB73/2 and D. solani IFB102 on (B) 25*ml *of 0.5x B-medium with 0.5% of agar and on (D) 7.5*ml *of 0.5x B-medium with 0.7% of agar. Red dots indicate inoculation point of IFB102 and green dots indicate inoculation point of MB73/2*

When grown in monoculture *D. solani* exhibits the total inhibition of swarming motility at the tested conditions (Fig. 20 A-C). However, the close presence of *B. subtilis* MB73/2 is able to trigger the directional escape of *D. solani* on 25ml of B-medium but not on media containing a higher concentration of agar (Fig. 20 B-D).

The volume of the medium in the plate determines a delay in the swarming-lag in both species. Consequently, MB73/2 approaches IFB102 when bacteria have already reached a high cell density at the inoculation point. Nonetheless, upon sensing the presence of *B.*

subtilis, a subset of *D. solani* initiates the formation of swarming tendrils and escapes in the opposite direction to MB73/2. This delay in directional escape results in a densely populated central colony with a visibly distinct inhibition zone.

The volume of the medium plays a crucial role in the accumulation of AHLs in the proximity of the cells, impairing the detection of AHLs and consequently the swarming motility of *D. solani* in plates containing 25ml of medium (Gatta et al., 2022). However, in the interaction assay, the presence of *B. subtilis* is able to trigger the directional swarming of *D. solani* in conditions that prevents swarming in the monoculture. This finding raises the possibility that motility and directional escaping in *D. solani* may be initiated by both quorum sensing and a danger-sensing mechanism in response to unidentified factors secreted by *B. subtilis*. A similar mechanism seems to induce exploratory motility in *Pseudomonas aeruginosa* in the presence of *S. aureus*. According to the authors, induced mobility is driven by secretion of surfactants in conjunction with antimicrobial factors. Presence of surfactin produced by MB73/2 may facilitate the swarming motility of *D. solani* by releasing the surface tension and simultaneously by facilitating the formation of membrane pores resulting in enhanced permeability to quorum sensing molecules. Role of surfactin in this antagonistic interaction will be discussed in next section.

Surface motility induction has also been observed in co-inoculation studies involving *PaeniBacillus vortex* and *Xanthomonas perforans*. In such instances, *X. perforans* effectively utilizes *P. vortex* rafts, enhancing its own motility by growing atop them. Furthermore, when bacteria are co-inoculated on the same plate but placed at a distance from each other, *X. perforans* exhibits directional swarming towards *P. vortex*, a behaviour not observed in single cultures where it remains at the point of inoculation (Hagai et al., 2014). These findings represent a clear example of the capacity for bacteria directional movement towards other species, which can confer benefits for their dispersal. In contrast, in scenarios like the MB73/2 vs IFB102 interaction, bacteria demonstrate movement in the opposite direction as a defensive response to factors that could affect their survival. Therefore, the induction of motility and directional movement may be more prevalent in natural environments than currently recognized.

In laboratory conditions, the mere presence of antagonistic species may not be sufficient to induce swarming motility if the surface itself presents physical impediments to such movement. In fact, IFB102 struggles to effectively escape from *B. subtilis* in media containing 0.7% agar. The hard surface impedes swarming, even in the presence of surfactin. Therefore, bacteria are unable to respond to the presence of *B. subtilis* by directional movement and rely solely on secreting antimicrobial compounds to prevent *B. subtilis* from approaching. Even without engaging in directional escaping, it is evident that bacteria tend to proliferate in the area diametrically opposite to *B. subtilis*.

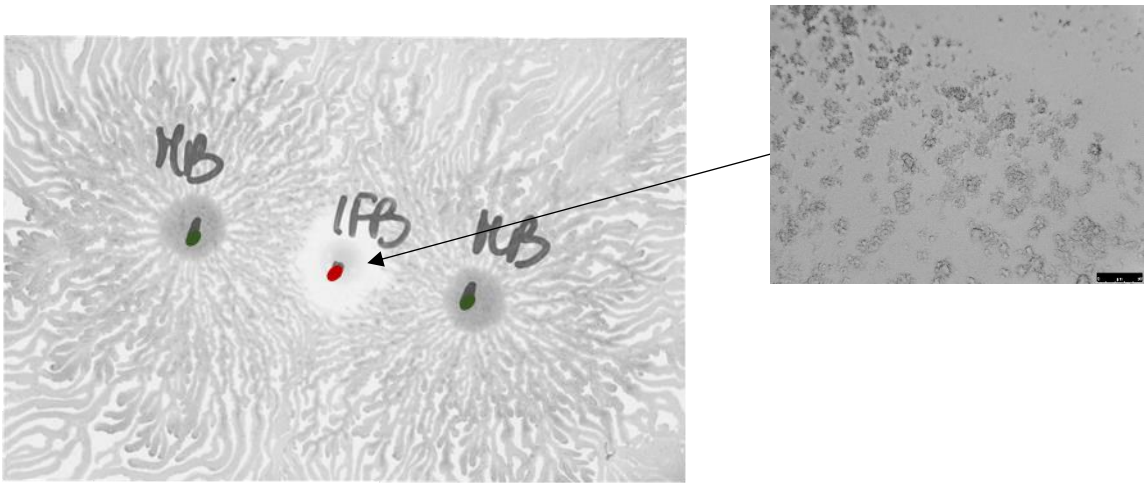

Figure 21- Swarming interaction of B. subtilis MB73/2 and D. solani IFB102 on 0.5x B-medium with 0.5% of agar. Bacteria are inoculated at a distance of 0.5 cm. The red dot indicates the point of inoculation od D. solani. Arrow points to the formation of cell lysis zones within D. solani colony. (B) Observation under confocal microscope of the cell lysis zones indicating presence of cell debris on the medium.

Escape serves as an effective strategy for evading the antimicrobial compounds secreted by *B. subtilis*. Limiting the directional escape, bacteria are unable to avoid the antimicrobials and are consequently subjected to lysis by *B. subtilis*. To validate this hypothesis, we inoculated MB73/2 and IFB102 on B-medium under conditions that allow the swarming of *D. solani* (7.5 ml 0.5x B-medium with 0.5% agar). However, we ensured that there was no opportunity for *D. solani* to escape by inoculating MB73/2 on both sides of IF102 at a distance of 0.5 cm. In these conditions, *D. solani* had insufficient time to initiate swarming motility, as upon exiting the lag phase, *B. subtilis* had already surrounded it. Consequently, following the formation of the inhibition zone, it became evident that the colony of *D. solani* began to undergo lysis, leaving only cell debris on the medium (Fig. 15). Hence, directional movement emerges as the sole effective strategy against the antimicrobials secreted by *B. subtilis*. While the formation of the inhibition zone prevents bacterial advancement, the antimicrobials persist in the medium, exerting their effect even in the absence of direct physical contact.

8.3 Time of inoculation of *D. solani* IFB102 and *B. subtilis* MB73/2 influences the interaction phenotype

The dynamic interaction between *D. solani* IFB102 and *B. subtilis* MB73/2 relies heavily on the ability of bacteria to secrete and react to antimicrobial compounds dispersed within the medium. Consequently, the duration of time that bacteria spend growing on the medium and their respective cell densities may emerge as crucial factors influencing how bacteria interact with one another. We conducted a swarming interaction assay using 0.5x B-medium with 0.5% agar, where *B. subtilis* MB73/2 was inoculated at various time points following the initial inoculation of *D. solani* IFB102 (t0, t4, t6, t8). Subsequently, we analysed the resulting phenotypes to assess the dynamics of their interaction.

When both bacteria are inoculated simultaneously, we observe the formation of an inhibition zone along with directional escape of *D. solani*. However, the dynamics of their interaction change when *B. subtilis* is inoculated later than *D. solani*. For instance, if *B. subtilis* is introduced 4 hours after *D. solani*, IFB102 has already colonized half of the plate, resulting in a visible inhibition zone but less evident directional escape. Moreover, when *B. subtilis* is introduced 6 or 8 hours after *D. solani*, IFB102 effectively colonizes the plate, confining *B. subtilis* to a limited area. In these cases, a strong inhibition zone is apparent, but directional escape is not observed.

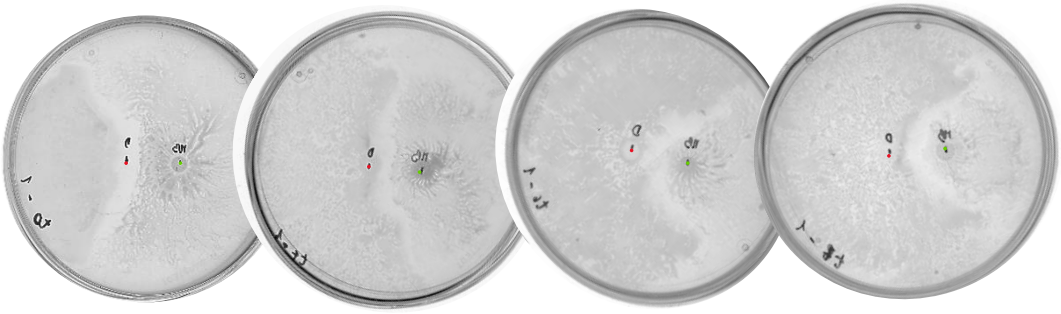

Figure 22- B. subtilis MB73/2 and D. solani IFB102 swarming interaction when B. subtilis is inoculated at different time points upon inoculation of D. solani. From left to right plates show interaction when B. subtilis is inoculated simultaneously with D. solani, or after 4, 6 and 8 hours. Red dots mark the point of inoculation of D. solani. Green dots mark inoculation points of B. subtilis.

These observations are consistent with our hypothesis that the antimicrobial compounds of *B. subtilis* are only secreted upon interaction. Thus, a certain amount of time is required to reach a sufficient population density, sense the presence of the antagonistic species, and subsequently produce and secrete the antimicrobial compounds. By reducing the duration of interaction and allowing *D. solani* to grow independently, *B. subtilis* has limited opportunity to impede the growth and motility of *D. solani*. Consequently, the

antimicrobial compounds secreted by IFB102 primarily contribute to the formation of a robust inhibition zone.

The influence of inoculation time on antagonistic interactions is not a novel concept and has been previously explored in several studies. These investigations have revealed that delayed inoculation can intensify antagonistic interactions or unveil positive interactions, primarily due to variations in population density at the time of interaction (Bashan 1986; Gonzalo et al., 2019). In our specific case, we hypothesize that the duration of exposure to the antagonist species, the presence of the antagonist, and the population ratio play crucial roles in determining the outcome of this prey-predator interaction. Further experiments have been conducted to verify this hypothesis.

8.4 The antimicrobial surfactin is required to directional escape of *D. solani* IFB102

When it comes to swarming motility, surfactants represent the most interesting molecules due to their capacity to alter surface tension, thereby enabling bacteria to swarm effectively on solid surfaces. *B. subtilis* produces surfactin, known for being the most powerful biosurfactant, as it is highly effective even at extremely low concentrations.

In *B. subtilis* the *srfA* operon-*sfp* gene cluster assumes the key role in surfactin production. The Sfp protein (4-phosphopantetheinyl transferase) is essential for surfactin synthesis. It facilitates the transfer of the 4′-phosphopantetheinyl moiety from coenzyme A to the serine residue of the peptidyl carrier protein (PCP) module, thereby activating surfactin synthesis (Reuter et a., 1999). However, in *B. subtilis* 168, the *sfp* gene harbours an internal termination codon, leading to the production of a truncated and nonfunctional Sfp protein.

Therefore, the laboratory strain *B. subtilis* 168N is not able to swarm in laboratory conditions. Similarly, we constructed a mutant strain of *B. subtilis* MB73/2 by inactivation of the *sfp* gene which was disrupted with pMutin4 by a single-crossover event (Campbell-type integration). A chromosomal fragment from the *sfp* gene was amplified by PCR and cloned into pMutin4 vector. The resulted mutant shows no production of srufactin, confirmed by drop-collapsing assay (Jain., 1991). At the same time, we obtained a *B. subtilis* 168N mutant with reconstituted *sfp* gene from Prof. Michal Obuchowski library.

The surfactin deficient strains *B. subtilis* 168 and *B. subtilis* MB73/2 *sfp*- were tested to evaluate the influence of surfactin on the interaction between *B. subtilis* and *D. solani*. The surfactin producing strains (MB73/2 and 168 *sfp*+) and the non-surfactin producing strains (168 and MB73/2 *sfp*-) exhibit a very different phenotype when it comes to swarming interactions.

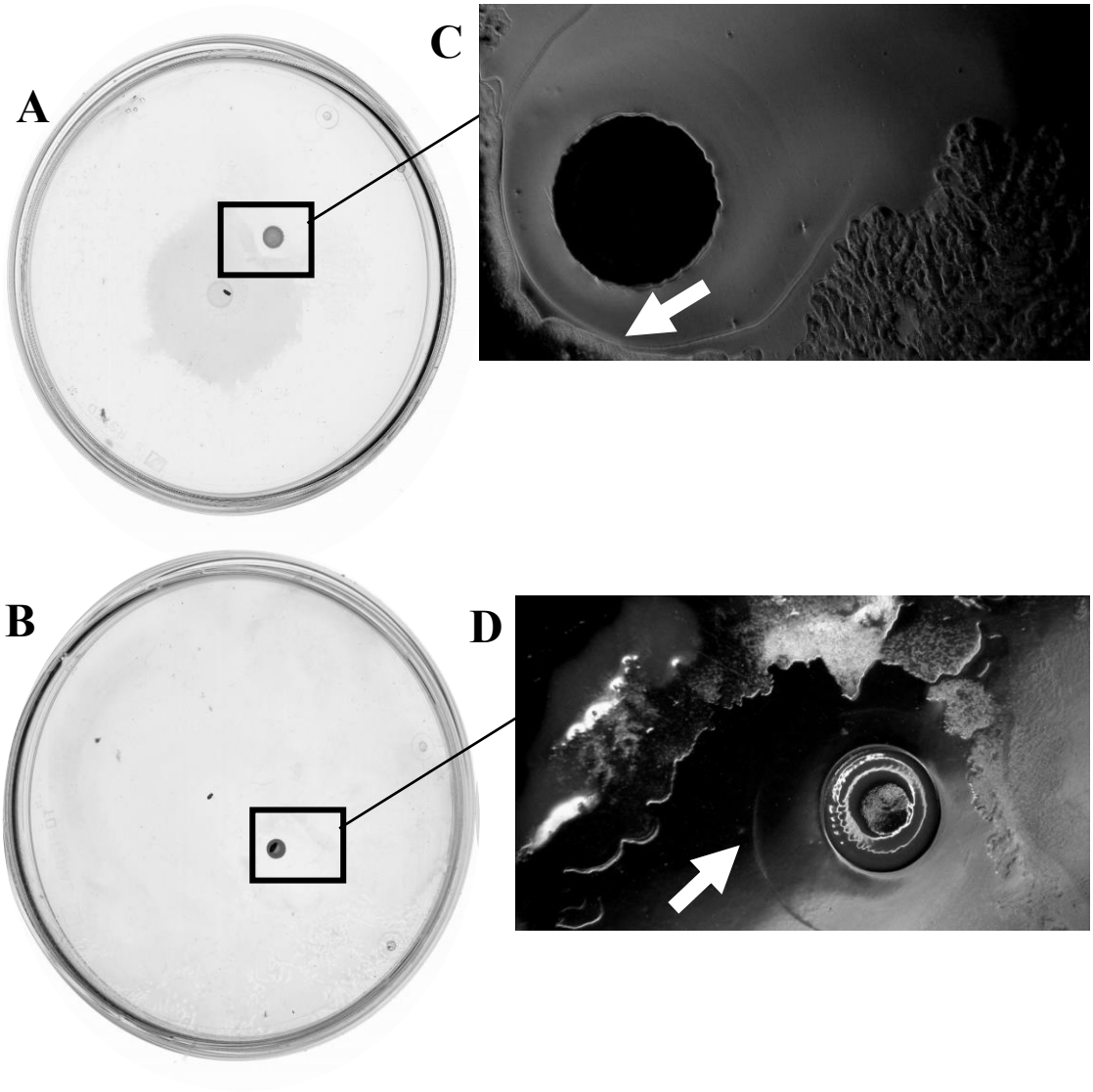

Figure 23- Swarming interaction of D. solani IFB102 and (A) B. subtilis 168N or (B) B. subtilis MB73/2 sfp-. (C) Interaction zone between D. solani IFB102 and B. subtilis 168N. (D) Interaction zone between D. solani IFB102 and B. subtilis MB73/2 sfp-. Arrows point to the definition of an inhibition ring.

D. solani IFB102 and mutants of *B. subtilis* were inoculated on the same swarming plate containing 0.5x B-medium with 0.5% of agar, with a separation distance of 1.5 cm. In the absence of surfactin, *D. solani* exhibits independent growth and swarming in all directions until it encounters *B. subtilis*, resulting in the formation of a prominent inhibition zone. However, only the advancement of the swarming front is inhibited, with no evident directional movement or escape. Upon closer examination at the point of interaction, it

becomes apparent that *B. subtilis* secretes some compounds that diffuse into the medium, causing a change in light refraction and forming a visible *ring* (Fig. 23 C-D). This *ring* delineates the confining zone that *D. solani* is unable to penetrate. The observed phenotype remains consistent across both mutant strains, *B. subtilis* 168N and *B. subtilis* MB73/2 *sfp*-, suggesting that both the environmental and laboratory strain possess equivalent abilities to inhibit *D. solani*. Remarkably, the formation of the inhibition *ring* is exclusively evident at the front surrounding the area where *B. subtilis* contacts *D. solani*. This observation suggests that bacteria commence secreting the compound only upon sensing the presence of the antagonist.

On the other hand, interaction of *D. solani* IFB102 with *B. subtilis* 168 *sfp*+ closely resembles the phenotype observed in the interaction with the environmental strain. This interaction results in the formation of an inhibition zone and the directional escape of *D. solani* (Fig. 24).

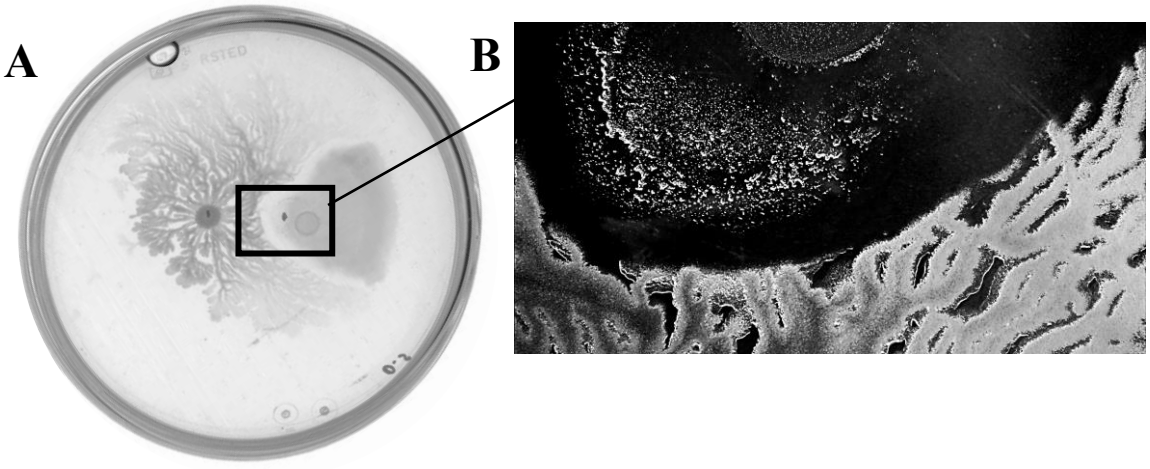

Figure 24- (A) Swarming interaction of D. solani IFB102 and B. subtilis 168N sfp+ on 0.5x B-medium containing 0.5% of agar. (B) Inhibition zone appearing between the interacting bacteria and D. solani IFB102 translocation from the point of inoculation.

Surfactin is renowned for its broad spectrum of bioactive properties, including antibacterial, antifungal, antiviral, anticancer, and anti-mycoplasma activities. Furthermore, it acts as an anti-adhesive agent against pathogenic bacteria, an insecticide, and a plant defense elicitor (Chen et al., 2022). The mechanism of action is not well elucidated, but it is believed that this peptide targets microbial cell membranes, leading to the formation of pores and eventually cell lysis. Therefore, we wanted to investigate if surfactin itself can affect bacteria growth and cause cell lysis of *D. solani* IFB102.

We conducted a swarming assay of *D. solani* IFB102 on plates supplemented with: (Fig. 25 A) 5x concentrated supernatant of *B. subtilis* MB73/2 and (Fig. 25 B) 20 µl of synthetic surfactin (Sigma-Aldrich) (10 mg/ml). Interestingly, the presence of surfactin in the medium did not adversely affect the growth rate of *D. solani* in either scenario. In contrast, surfactin appeared to enhance both the growth and swarming motility of the bacterium. Surfactin positive impact on bacterial interactions is further exemplified in the interaction between *B. subtilis* 3610, a surfactin producer, and *P. dendritiformis* (Luzzatto-Knaan et al., 2019). Upon interaction, the authors reported that rather than functioning as an antimicrobial compound, surfactin acted as an attractant for *P. dendritiformis* toward *B. subtilis*. This phenomenon was interpreted as a mechanism for *B. subtilis* to attract other plant growth-promoting rhizobacteria (PGPR). Essentially, surfactin may serve as clever strategy in which *B. subtilis* sends out a signal to recruit or repel other organisms from its ecological niche, thereby establishing more favourable conditions.

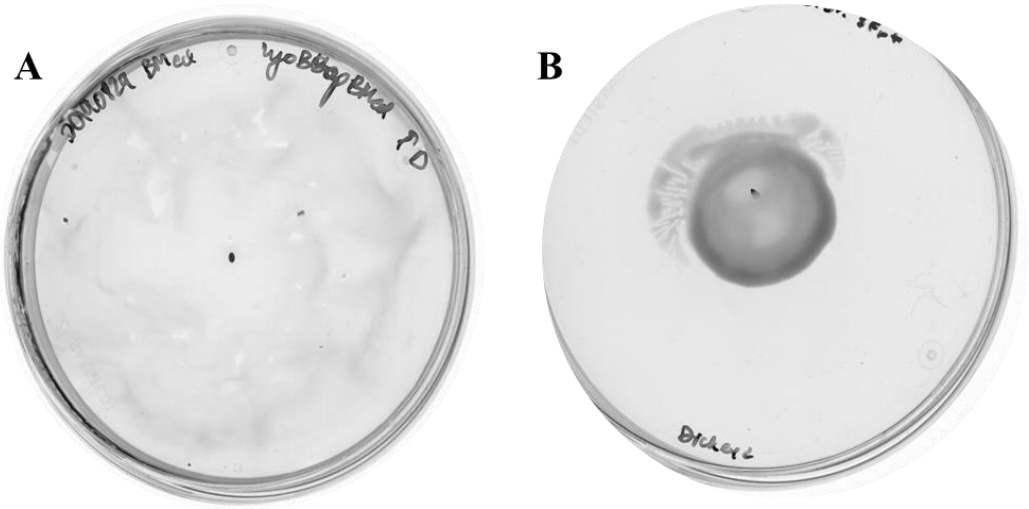

Figure 25 -- (A) Swarming of D. solani IFB102 on 0.5x B-medium with 0.5% of agar supplemented with 5x concentrated B. subtilis MB73/2 CFS (B) Swarming of D. solani on 0.5x B-medium with 0.5% of agar. 20µl of synthetic surfactin were spotted on the medium before bacteria inoculation.

The presence of surfactin is essential for the directional escape of *D. solani*. In its absence, *D. solani* swarms toward *B. subtilis*, highlighting the crucial role of surfactin in influencing bacterial movement. Beside reducing surface tension, surfactin interacts with bacterial membranes, potentially enhancing permeability to quorum sensing signals, thereby leading to increased swarming motility. Therefore, the antimicrobial activity of MB73/2 against IFB102 cannot solely be attributed to the presence of surfactin, suggesting the involvement of other antimicrobial compounds.

These observations led us to hypothesize that surfactin is essential to (i) facilitate the diffusion of antimicrobial compounds produced by MB73/2 in the medium and (ii)

enhance membrane permeability to quorum sensing molecules, thereby enabling directional escaping in response to the perceived antimicrobial compounds.

8.5 Known antimicrobial compounds secreted by *B. subtilis* MB73/2 do not affect the swarming and growth of *D. solani* IFB102

The biocontrol potential of *B. subtilis* lies in its capacity to secrete a diverse array of antimicrobial compounds. *B. subtilis* sp. produce over two dozen antibiotics with approximately 10% of the annotated ORFs within the *B. subtilis* genome designated to antibiotic production (genes involved in the whole pathways occupy 350kb: NRPSs 200 kb, PKs 76 kb, lantibiotics 50kb and other antibiotic requiring over 20 kb). However, single strains typically produce only a subset of the diverse array of antimicrobial compounds mentioned, generating high diversity in antimicrobial activity within the group (Stein., 2005).

In our investigation, we aimed to identify the antimicrobial compounds secreted by *B. subtilis* strain MB73/2 responsible for the two specific phenomena observed during its interaction with *Dickeya solani*: the formation of an inhibition zone and the directional escaping.

Due to the complexity of the observed interaction, we speculated a multi-factorial nature of the observed phenotype. Therefore, we initially relied on random mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) to generate a library of MB73/2 mutants, by inducing random point mutations in the genomic DNA.

Following mutagenesis, we screened the mutant library for strains exhibiting alterations in the phenotype of interest. Given the technical challenges associated with performing swarming assays under laboratory conditions, we adopted a two-step screening approach. In the initial screening phase, mutants were assessed using a spot-on-lawn antimicrobial assay. This method allowed the rapid screening of over thousands of mutants generated by MNNG mutagenesis, by spotting them onto agar plates seeded with *D. solani* IFB102. From this extensive screening, only 50 mutants, displaying either no inhibition zone or reduced inhibition zone in comparison to the wild-type strain, were selected for the swarming interaction assay.

Mutants were further classified into two distinct subsets (surfactant producer and non-surfactant producer) based on their ability to produce surfactin which was assessed by drop-collapsing assay. As expected, surfactant-producing mutants demonstrated robust swarming motility on 0.5x B-medium with 0.5% agar. In contrast, non-surfactant producers showed impaired swarming motility. Surprisingly, upon interaction with *Dickeya solani* IFB102, neither the surfactant-producing mutants nor the non-surfactant-producing mutants exhibited significant differences in phenotype compared to the wild-type strains. Surfactant-producing mutants displayed interaction dynamics similar to those of the parental strain *Bacillus subtilis* MB73/2, while non-surfactant-producing mutants behaved similarly to the laboratory strain 168.

Despite the large library of screened mutants, the lack of discernible differences in interaction dynamics raised questions about the effectiveness of our mutagenesis protocol in inducing relevant genetic alterations and prompted us to reevaluate the efficacy of our chemical mutagenesis approach. Therefore, we selected nine mutants representing a spectrum of phenotypic variations, spanning both surfactin producer and non-surfactin producer subsets that were subjected to whole genome sequencing.

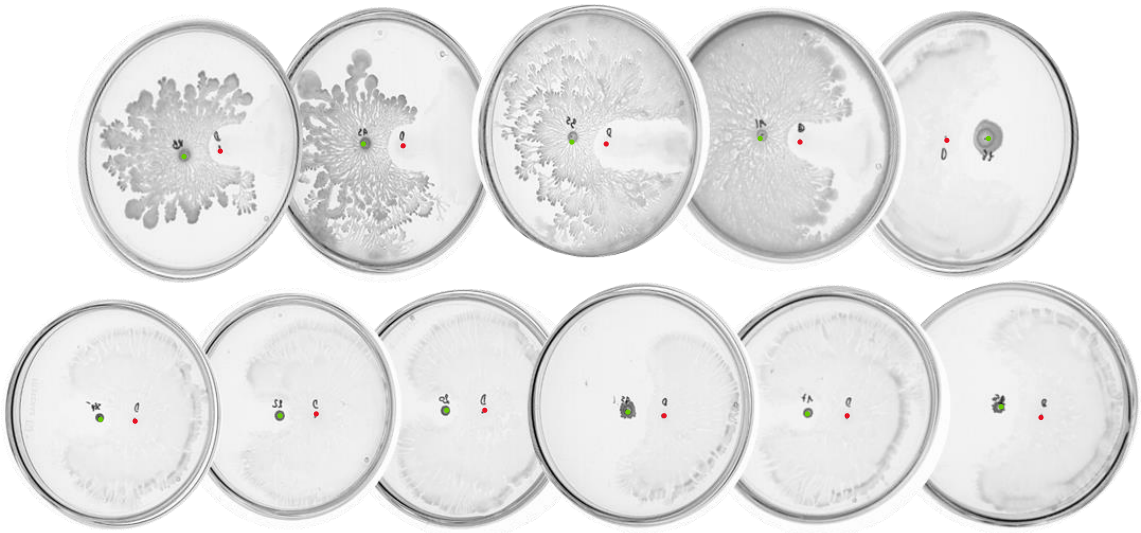

Figure 26- Swarming interaction assay of MB73/2 mutant strains against D. solani IFB102. (A) Mutants of MB73/2 representatives of the subset of surfactin-producer strains. From left to right: MB73/2, 45, 25, 41, 37. (B) Mutants of MB73/2 representatives of the surfactin deficient strains. From left to right: MB73/2 sfp-, 22, 20, 451, 17, 35.

The genomes of the nine mutants of MB73/2 were sequenced by Genomed on the MiSeq platform (Illumina). *Bacillus subtilis* MB73/2 shows a high genomic identity with the laboratory strain 168N, except for a distinct prophage region of 20435bp not present in the genome of MB73/2 (Iwanicki, unpublished). Mutant strains were aligned to the reference genome of *B. subtilis* 168 using BLAST RingImageGenerator which confirmed 100% identity with the reference genome. This provides compelling evidence of the fidelity of the chemical mutagenesis, ensuring minimal off-target effects on the genomic integrity of the mutants.

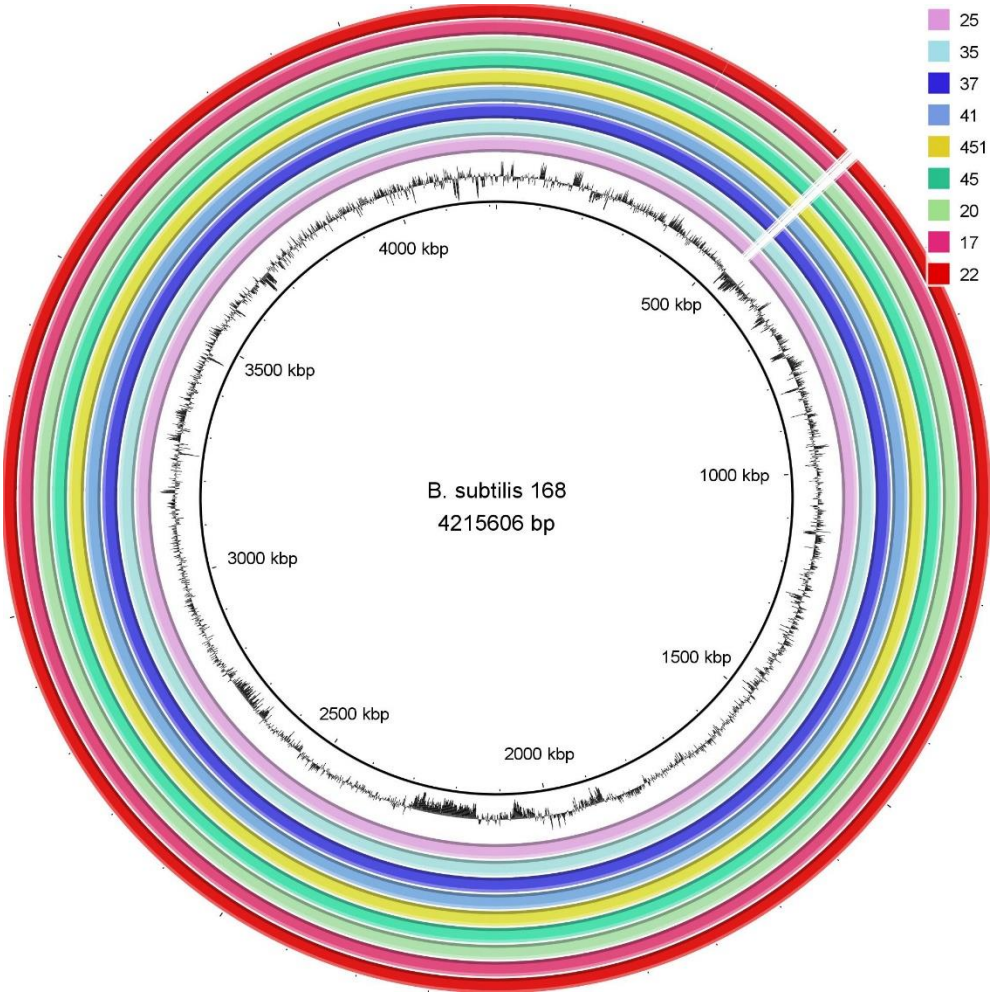

Figure 27 - Sequence alignment using BLAST RingImageGenerator. Consensus sequences of the mutant strains of MB73/2 were aligned on the reference genome of B. subtilis 168. Gaps show absence of 20435bp prophage region in the analysed genomes.

Subsequently, we used megablast to identify substitutions and gaps between the mutants and genome of MB73/2. However, our analysis yielded unexpected results, revealing over 100 substitutions and gaps in each mutant strain across several genes.

Despite our efforts, we were unable to obtain a mutant displaying the desired phenotype. However, even if we had succeeded in identifying such a mutant, the genetic variations

identified would have posed significant challenges for downstream analysis to pinpoint specific mutations associated with the observed phenotypic variations. These observations prompted us to reconsider the suitability of MNNG mutagenesis to generate mutants.

Therefore, we made the decision to use transposon mutagenesis using the pMarB plasmid, to generate new mutants. Unlike chemical mutagenesis, transposon insertion enables random but limited disruptions of genomic loci, facilitating the downstream analysis. The pMarB plasmid harbours a transposable element (TnYLB-1), consisting of a Kanamycin resistance cassette flanked by Himar1-recognized inverse terminal repeats, along with a temperature-sensitive replicon and Erythromycin resistance gene from pE194ts (Le Breton et al., 2006). The construction of the MB73/2-pMarB strain posed initial challenges due to the reduced competence of the environmental MB73/2 strain. To overcome this limitation, we supplemented the starvation medium with 30% filtered Cell-Free Supernatant (CFS) from *Bacillus subtilis* 168 grown under starvation. The rationale behind this strategy was to enhance the concentration of ComX present in the medium, thereby improving the competence of our environmental strain. We successfully obtained the MB73/2-pMarB strain using this approach.

A library of several hundred mutants were generated by transposon mutagenesis. Following the two-step screening approach, we identified 74 strains for the swarming interaction assay. However, results remained inconclusive with none of the generating mutants exhibiting a different phenotype from the wild type in terms of social interaction with *D. solani* IFB102. The inconclusive findings underscore the complexity of microbial interactions and corroborate our initial hypothesis regarding the multifactorial nature of the observed phenotype, suggesting that different genes may contribute to the complex dynamics observed during social interactions.

In ultimate analysis, we decided to investigate the role of known antimicrobial compounds produced by *B. subtilis* on the antagonistic interaction with *D. solani*. To facilitate this investigation, we acquired a library of single gene deletion mutants of *B. subtilis* 168 from the National BioResource Project (NIG, Japan). The BKE library comprises 3968 mutants, each featuring a single-gene deletion. These mutants were engineered with the Erythromycin resistance cassette replacing the entire open reading frame (ORF) of the targeted gene, while preserving the start and stop codons. The

resistance cassette lacks a terminator, ensuring that its presence does not interfere with the expression of downstream genes. Selection of the mutants was based on the list of genes involved in the biosynthesis of antimicrobial compounds presented by SubtiWiki (http://subtiwiki.uni-goettingen.de/v4/category?id=SW.2.6.6.1).

The swarming assay of the single gene deletion mutants failed to reveal any noticeable differences in phenotype when compared to the wild-type strain *B. subtilis* 168. Surprisingly, all mutants displayed inhibition zones on agar plates, closely resembling the behaviour of the wild type. Furthermore, this lack of discernible differences in phenotype suggests that known antimicrobial compounds such as fengicin, iturin, pilpastin, and other polyketides (PKs) do not play a significant role in the observed phenotype. This prompts us to consider the potential involvement of novel, unidentified antimicrobial compounds in mediating this interaction, highlighting the need for further exploration and investigation.

8.6 Spo0A regulation is not involved in the synthesis of antimicrobial compounds against *D. solani*

Our findings have directed our attention towards investigating a multifactorial response as the underlying mechanism of the antagonistic interaction. Consequently, we sought to investigate the involvement of master regulators in the observed antagonistic behaviour.

In *B. subtilis*, the synthesis of NRPs is under repression of the AbrB regulator, which is negatively controlled by phosphorylated Spo0A. P-Spo0A, in turn, activates the transcription of genes involved in NRP synthesis by releasing the repression exerted by AbrB. Spo0A is a transcription factor that is considered the master regulator of sporulation in *B. subtilis*. When phosphorylated, Spo0A binds to specific DNA sequences known as '0A-boxes,' thereby directly regulating the expression of 121 genes leading to sporulation (Liu et al., 2003). However, levels of P-Spo0A are not constant during the growth and P-Spo0A gradually accumulates in the cells making sure that other responses to nutrient depletion, including lysis of siblings and antagonistic species, can be activated before committing to sporulation. Therefore, we wanted to elucidate the role and interplay of AbrB and Spo0A in the antagonism with *D. solani*.

Unfortunately, the AbrB deletion mutant present in the BKE library was not able to grow on synthetic B-medium (data not shown). Consequently, we decided to focus on the Spo0A regulation and constructed a mutant of MB73/2 by inactivation of *spo0A*. The

spo0A gene was disrupted with pMutin4 by a single-crossover event. A chromosomal fragment from the *spo0A* gene was amplified by PCR and cloned into pMutin4. The plasmid was transferred by protoplast electroporation. The resulted mutant was analysed by NGS sequencing and sporulation assay, revealing proper integration of pMutin4 in the chromosome and absence of spores.

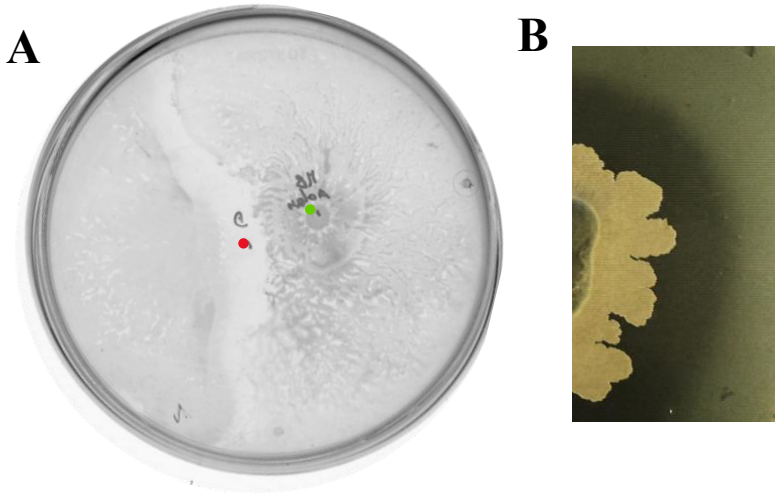

Figure 28 - Interaction between MB73/2 spo0A- and D. solani IFB102.(A) Interaction on semi-solid 0.5x B-medium with 0.5% agar. Red dot indicates the point of inoculation of D. solani. Green dot indicates the point of inoculation of B. subtilis. (B) Interaction on solid agar with spot on lawn assay. D. solani is inoculated in the medium with B. subtilis MB73/2 spo0A- inoculated on top of it.

The MB73/2 *spo0A*- mutant was investigated in its interaction with *D. solani* IFB102 on 0.5x B-medium with 0.5% agar, following the swarming interaction assay. Additionally, we tested the ability of MB73/2 *spo0A*- to inhibit the growth of *D. solani* IFB102 with the spot-on-lawn assay. Obtained results, showed no significant differences between the *spo0A*- and the wild type MB73/2, with formation of inhibition zone on the solid agar (Fig. 28 B) and presence of the inhibition zone followed by directional escaping upon interaction on semi-solid medium (Fig. 28 A).

Spo0A is also known for being associated with surfactin production and several studies have documented the loss of surfactin in *spo0A*- mutants (Rosier et al., 2023). However, the exhibited swarming motility suggests that our strain is not impaired in the production of surfactin. Although there appears to be a delay in the directional escaping of *D. solani*, pointing to a potential reduction in surfactin levels. These findings are consistent with the results proposed by Sun and colleagues (2021) which proved that surfactin production in an *B. amyloquefaciens spo0A*- mutant was reduced but still detectable. It is possible that other regulatory mechanisms influence the levels of AbrB in the cells, leading to the

transcription of the genes involved in the NRPs synthesis, including *sfp*. Therefore, by inactivation of *spo0A* we cannot exclude that other NRPs may be involved in the antagonism between *B. subtilis* and *D. solani*.

It is worth noting that Spo0A phosphorylation plays an important role in the production of antimicrobial factors beyond NRPs, including ribosomally synthesized peptides, enzymes, and proteases. Specifically, Spo0A controls the production of subtilin and the sporulation killing factor (skf). Therefore, our results led us to exclude the role of subtilin and skf in the observed antagonism, suggesting that Spo0A does not regulate antagonistic interaction with *D. solani*.

8.7 *B. subtilis* and *D. solani* antagonism is a surface-dependent interaction

The analysis of whole genome sequences and the generation of mutant strains have yet to fully elucidate the nature of the antimicrobial compounds secreted during the interaction between *B. subtilis* and *D. solani*. However, observations from their interaction on swarming plates suggest that the secretion of antimicrobials occurs specifically upon sensing the presence of the antagonist. This inference finds support in the formation of a ring of inhibition only at the forefront of the interaction with *D. solani* (Fig. X). Our results also indicate an exchange of signalling molecules among the interacting species which is responsible for the escaping of *D. solani* even in the absence of physical contact among the bacteria.

To confirm whether *B. subtilis* secretes antimicrobials only upon interaction, our objective was to assess the antimicrobial activity of the cell-free supernatant (CFS) from MB73/2 on the growth and swarming behaviour of *D. solani*. Initially, we investigated the effect of *B. subtilis* MB73/2 CFS on the growth of *D. solani* IFB102 by measuring the OD600 of planktonic growth over an 18-hour period. However, we did not observe any significant suppression of growth at any time point. To further explore this phenomenon, we lyophilized and concentrated the CFS to determine whether the concentration of antimicrobial compounds was too low to exert an effect. Surprisingly, even after supplementing the medium with 10x and 25x concentrated CFS, we did not observe any inhibitory effect on the growth of *D. solani*.

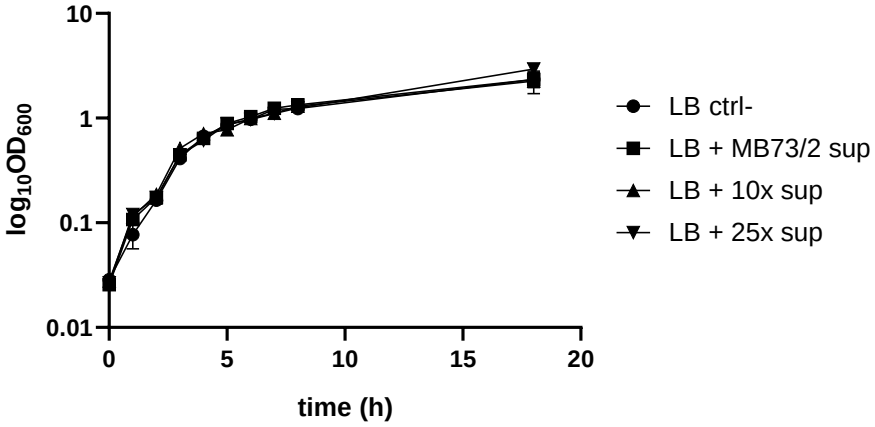

Figure 29- Growth curve of D. solani IFB102 in LB in comparison with the growth curve of D. solani IFB102 in LB supplemented with MB73/2 CFS and 10x or 25x concentrated CFS. OD600 was measured every hour for 8 hours. An endpoint measurement was taken after 18h.

Simultaneously, we assessed the effect of the supernatant on the swarming motility of *D. solani*. To simulate the conditions of the swarming assay interaction, we spotted 20 µl of MB73/2 CFS on the plate at a distance of 1.5 cm from the point of inoculation of *D. solani*. As anticipated, the CFS did not inhibit the swarming of *D. solani*; instead, we observed *D. solani* swarming towards the CFS inoculation point. It is worth noting that the CFS of MB73/2 was prepared in the rich medium LB. Therefore, it is plausible that *D. solani* swarms towards the CFS because it perceives it as a source of nutrients. Similarly, we supplemented the swarming medium with 1 ml of CFS and utilized it to evaluate the swarming motility of *D. solani*. As anticipated, the presence of surfactin in the CFS significantly enhanced swarming, acting as a genuine trigger for swarming rather than an inhibitor.

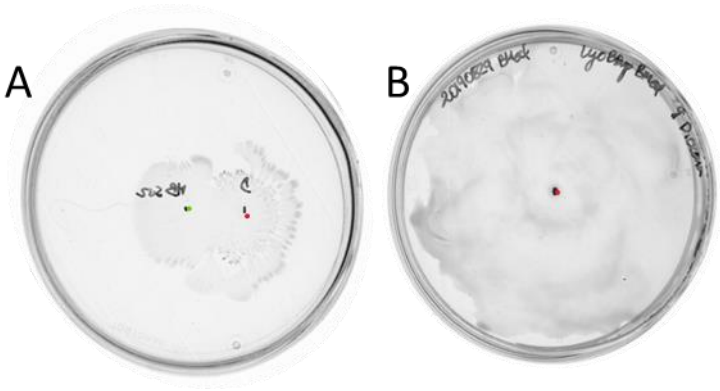

*Figure 30 - (A) Swarming of D. solani IFB102 on 0.5x B-medium with 0.5% of agar inoculated at 1.5 cm from 10ul of CFS of MB73/2. (B) Swarming of IFB102 on 0.5x B-medium with 0.5% of agar supplemented with 1*ml *of B. subtilis Mb73/3 CFS. The green dot indicates the CFS spotting point. The red dots indicate the point of inoculation of D. solani*

Several studies have reported the inhibitory capacity of *B. subtilis* CFS against a broad range of human and plant pathogens including *S. aureus* (Zhang et al., 2021), *Listeria monocytogenes* (Park et al., 2023), *Candida albicans* (Subramenium et al., 2018), B. dothidea (Fan et al., 2023). However, it is evident that the CFS of MB73/2 is completely ineffective against *D. solani*. While these results support our hypothesis of a sensing-response antimicrobial secretion, they also suggest the involvement of a much more intricate regulatory mechanism that activates the expression of genes only upon interaction.

The concept of silent genes involved in the biosynthesis of antimicrobial compounds is not novel and has emerged with the advancements in genomics. Modern microbial genomics have unveiled the presence of biosynthetic gene clusters that remain dormant or are not expressed in mono-cultures (Zhong et al., 2018). Consequently, co-culturing of bacteria has garnered increasing attention as a strategy to stimulate the production of secondary metabolites in a manner that more closely mimics the natural environment.

Therefore, we wanted to analyse the behaviour of *D. solani* in co-culture with MB73/2. In order to accurately count cells during co-culture in liquid medium, it was essential to have a resistant cassette in both the *D. solani* IFB102 and *B. subtilis* MB73/2 strains, allowing for selective growth on counting plates. However, our attempts at transforming IFB102 via electroporation and conjugation proved to be exceedingly challenging, resulting in failure to obtain any clones. As an alternative, we utilized a mCherry-tagged *D. solani* strain from the R. Czajowski collection, which harboured the erythromycin-resistant cassette. Concurrently, we employed the MB73/2-gfp tagged strain containing the spectinomycin-resistant cassette from our laboratory collection. The fluorescence of each species was confirmed under a confocal microscope It is important to note that we initially treated *D. solani* IPO2222 and IFB102 strains as complementary due to the close similarity revealed by whole genome sequencing analysis, which identified only 7 SNPs between them. However, upon closer examination, we discovered that strains IPO222 and IFB102 can differ in their interaction with *B. subtilis* MB73/2. The comparison between IPO2222 and IFB102 is further investigated in the next chapter.

The growth of erythromycin-resistant *D. solani* IPO2222 *mCherry* was analysed in both single-species and mixed-species (1:1 ratio) planktonic cultures over a period of 6 hours. The following day, a follow-up sample collection was conducted to confirm the presence

of living cells in the co-culture mixture (t18=6.7 x 108 CFU/ml). The results surprisingly indicate that despite the presence of the antagonistic *B. subtilis*, the growth of *D. solani* IPO222 *mCherry* in co-culture was not significantly affected compared to the control (Fig. 31).

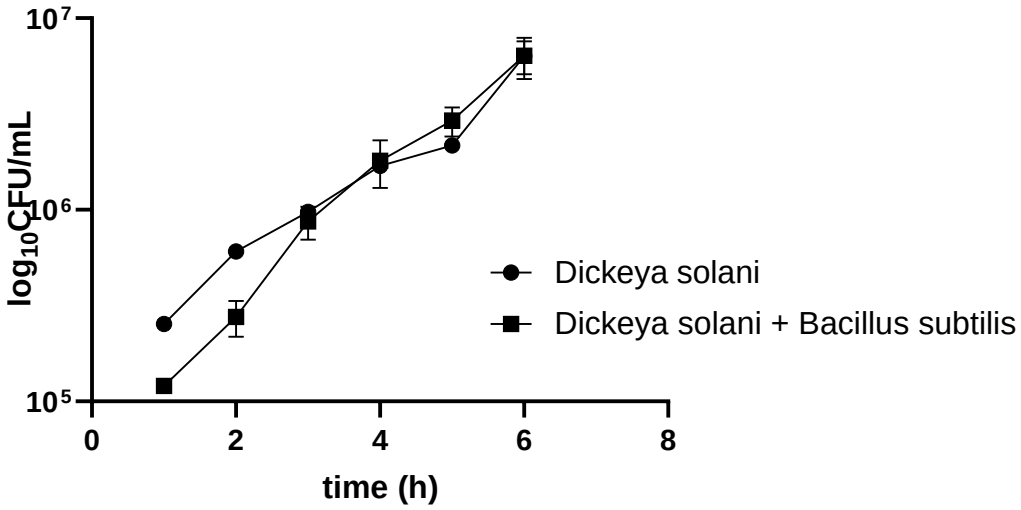

*Figure 31- Growth curve of D. solani IPO2222-mcherry in LB and in co-culture with MB73/2-gfp. Samples were collected every hour for six hours and CFU/*ml *were calculated from serial dilutions Error bars indicate standard deviation. The experiment was repeated twice with three technical replicates..*

To explore the impact of co-culture on swarming behaviour, a 2 µl sample of the liquid co-culture was spotted onto a swarming plate after 4 hours from the initiation of the mixed culture and incubated for the remaining 14hours. Unexpectedly, despite *D. solani* IPO2222 *mCherry* ability to grow planktonically when co-cultured in liquid medium, the transition to semi-solid medium resulted in complete growth inhibition and cell lysis, rendering IPO2222 *mCherry* undetectable upon observation under confocal microscope (Fig. 32).

These results suggest a radical shift in behaviour in response to the altered growth conditions. However, it must be considered that the change in growth conditions coincides with a transition from planktonic growth to sessile colonization. Indeed, gene expression changes when bacteria function as individual free-living cells compared to when they exhibit social behaviours and form community structures, such as in swarming motility. Moreover, liquid medium limits the occurrence of physical interactions among cells. First, it eliminates cell-surface interactions, which are prevalent in solid media where cells adhere to surfaces. Second, it minimizes cell–cell excluded volume interactions, which occur when cells are densely packed in confined spaces. Finally, liquid medium also reduces hydrodynamic interactions, a consequence of constant shaking, which tends to disrupt close proximity interactions between cells.

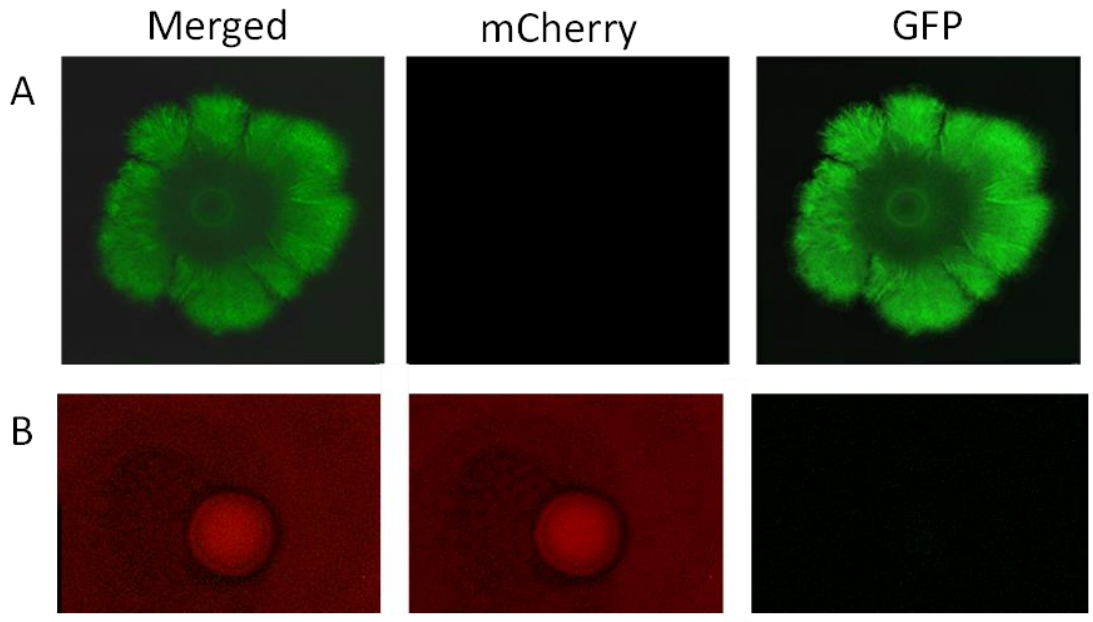

Figure 32 - (A) Swarming motility of IPO2222-mCherry and MB73/2-gfp co-cultured in LB for 4 hours and then inoculated on 0.5x B-medium with 0.5% of agar. Plate was observed under confocal microscope 18h upon inoculation. (B) Control sample - swarming motility of IPO2222 mCherry

Based on these observations, we hypothesize that distinct interactions take place on surfaces. Therefore, we repeated the co-culture assay directly on semi-solid medium for swarming motility. The differentially labelled strains were refreshed to OD600=0.03 and mixed in equal amount just prior to inoculation in the center of a swarming plate.

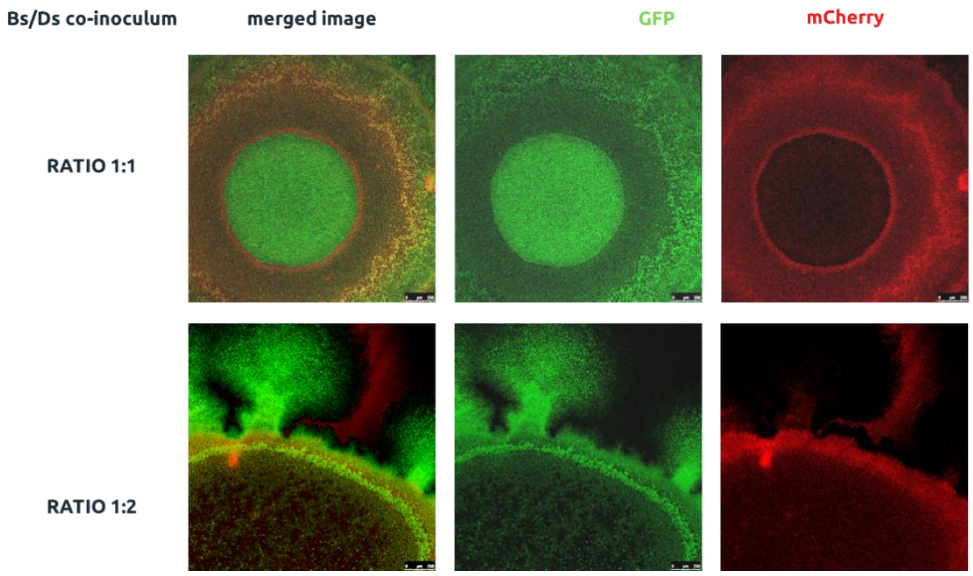

Figure 33- Swarming motility of IPO2222-mCherry co-inoculated with MB73/2-gfp. Bacteria were mixed just before inoculation with a MB73/2:IPO2222 ratio of 1:1 and 1:2. Plates were observed under confocal microscope 18hours after inoculation

At a macroscopic level, the two species display a unique swarming phenotype, appearing to form a single confluent swarm. However, fluorescent labelled strains allowed the detection of distinct swarming fronts, indicating that, on the colony level, the swarm is indeed heterogeneous. Specifically, the center of the swarming colony was predominantly colonized by *B. subtilis* MB73/2 *gfp* (green), whereas *D. solani* IPO2222 *mCherry* (red) was confined to an interaction zone located between the center of the colony (a) and the formation of the swarming tendrils (b). Within this zone, the two species coexist and mix, but the lower cell density suggests a form of antagonism. Indeed, *D. solani* is unable to penetrate the swarming tendrils produced by *B. subtilis* and remains confined to the interaction zone.

The low cell density within the interaction zone prompted us to investigate the interplay between the two colonies when IPO2222 *mCherry* was inoculated in a higher ratio. Interestingly, the change in the inoculum ratio determined a change in the physical separation between the two colonies. Specifically, *B. subtilis* failed to predominantly colonize the center of inoculation, which, on the other hand, appeared to be less populated, indicating the occurrence of antagonistic interactions. Moreover, under such conditions, *D. solani* was observed to form independent swarming tendrils. Notably, the two swarming tendrils remained distinct and the two bacterial species, despite originating from the same central colony, swarmed independently. A higher inoculum suggests that *D. solani* may achieve the threshold for quorum sensing-driven swarming motility sooner,

allowing secretion of antimicrobial compounds and independent motility. The segregation and heterogeneity of the swarming suggest a mechanism of self-recognition that allows bacteria to (i) recognize members of their species, (ii) engage into antagonistic interaction, (iii) swarm independently from their competitor.

Similar interaction has been reported in a recent study by Natan and colleagues (2022) where the authors investigated the interplay and segregation between *B. subtilis* and P. aeruginosa. The two species were found to swarm together forming a single colony but spatially heterogenous with no inhibition zone or demarcation line between the interacting species. Additionally, they also observed no antagonism in liquid co-culture, which was view by the authors as result of different gene expression in liquid culture and swarming.

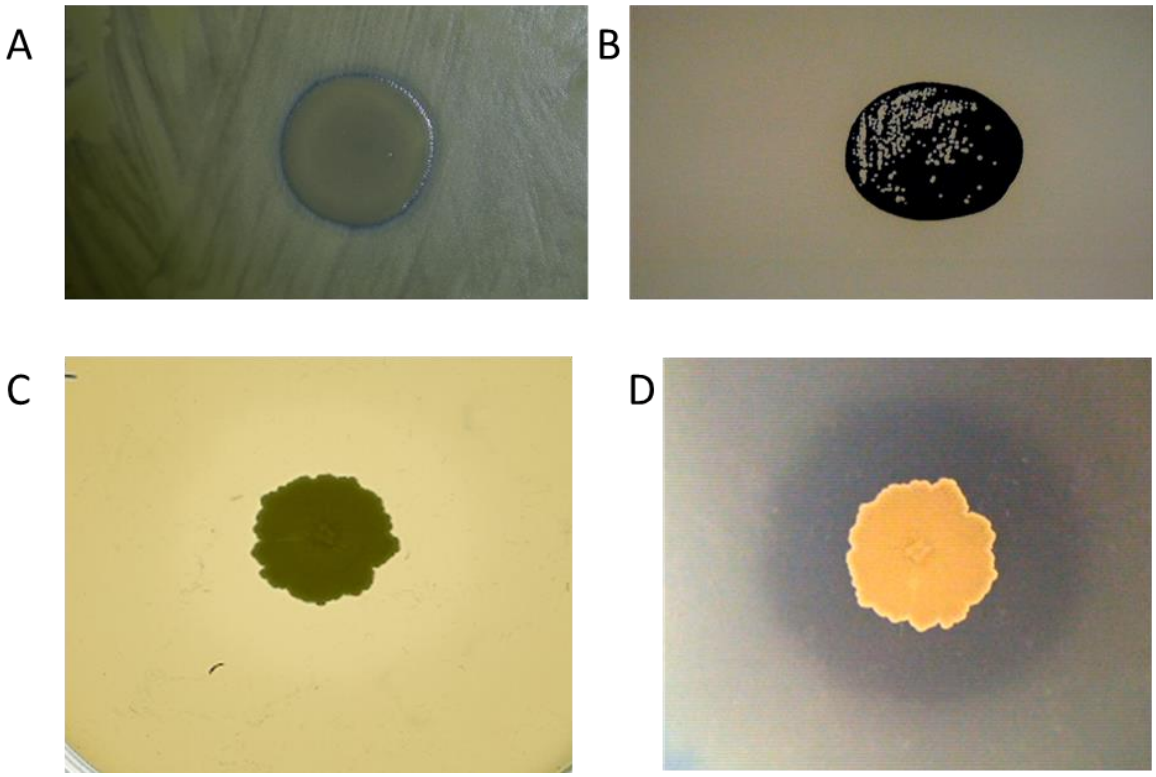

Figure 34- Interaction between IFB102 and MB73/2 on solid medium. (A) B. subtilis was spread over an LA plate and 20µl of D. solani overnight culture was inoculated on top of it. Inhibition zone was observed after 24h. (B) Plate observed after prolonged incubation to 48h. D. solani was lysed and only a few colonies of MB73/2 were able to grow in the area previously covered by D. solani. (C) IFB102 was inoculated into LA soft-agar plate and 20µl of MB73/2 were spotted on top of it. After 24h, no inhibition zone was visible. (D) Prolonging the incubation to 48h resulted in the formation of an inhibition zone.

Interestingly, they reported that the response to the antagonist's presence during interaction on solid agar exhibited a notable delay, which we also observed in the interaction between *D. solani* IFB102 and *B. subtilis*. Specifically, no inhibition zone was

discernible after 24 hours, when *B. subtilis* was spotted on an LB agar plate inoculated with *D. solani* IFB102 and only upon extending the incubation to 48 hours did the inhibition zone form. Conversely, when *B. subtilis* was spread across the medium and *D. solani* was centrally spotted, after 24 hours, both bacteria exhibited evident growth (Fig. 34), with an inhibition zone surrounding *D. solani*. Subsequently, after 48 hours, *D. solani* IFB102 was completely lysed, but only a few colonies of *B. subtilis* were able to emerge in the region previously occupied by *D. solani*. The emerging colonies were confirmed by PCR to be *B. subtilis* MB73/2. Consequently, they were isolated and tested against *D. solani*. Unfortunately, no difference in phenotype emerged from the interaction. These results confirm that (i) both interacting species secrete antimicrobial compounds; (ii) the antimicrobials are secreted upon sensing the antagonism and accumulate over time, causing a delay in the formation of the inhibition zone; (iii) an adaptative response may allow *D. solani* to initially resist to *B. subtilis*, until the accumulation of the antimicrobial is sufficient to inhibit the growth and cause the lysis of the bacterium.

8.8 *Bacillus subtilis* MB73/2 suppresses soft-rotting in in-vivo studies on potatoes inoculated with *D. solani* IFB102 and IPO222

To evaluate the effectiveness of the antagonistic *B. subtilis* MB73/2, we tested the ability of *D. solani* to induce soft-rot symptoms on potato slices in co-inoculum with *B. subtilis*. The experiment was conducted according to the dents in potato slices protocol widely reviewed in literature (Czajkowski et al., 2014) and with the contribution of Prof. Robert Czajkowski from the University of Gdansk.

For the assessment, potatoes underwent sterilization and were sliced into 1.5 cm deep sections. Subsequently, 1 cm diameter holes were created in the slices and filled with 100 µl of an overnight suspension of *D. solani*. Following this, *B. subtilis* overnight culture was added at a volume of 50 µl. After 96 hours of incubation, the slices were observed, and the diameter of the rotting tissue was measured to quantify the extent of soft-rot symptoms. Control samples were treated with water.

Our results (Fig. 35) suggest that presence of *B. subtilis* can significantly reduce the maceration ability of *D. solani*, thereby exerting a positive effect ($p < 0.01$) on preventing rot symptoms on potato slices. Notably, both the environmental strain MB73/2 and the

laboratory strain 168 show efficiently suppresses the soft rot caused by the *D. solani* wild type IPO2222 and by the environmental strain IFB102.

As complementary result to the mutants testing, we also decided to assess the protective

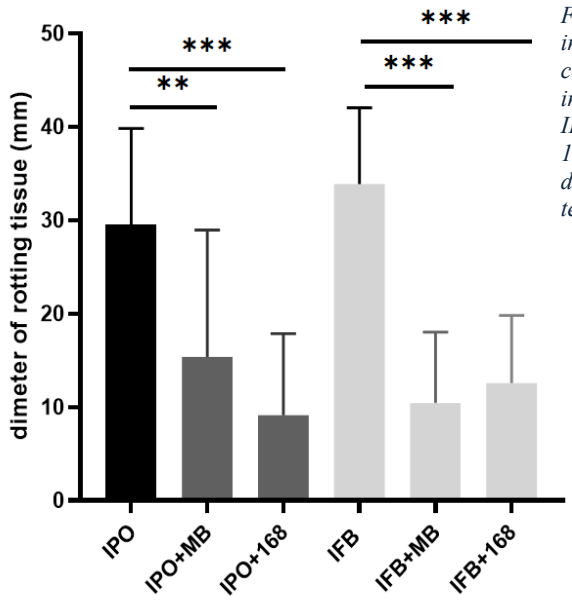

Figure 36- Diameter of rotting tissue (mm)in potato slices infected with Dickeya solani IPO2222 and IFB0102 in comparison with the diameter of rotting tissue in potato slices infected by Dickeya solani IPO222 or Dickeya solani IFB0102 in co-inoculum with B. subtilis MB73/2 or B. subtilis 168 96h after infection. Error bars represent standard deviation. The experiment was repeated two times with three technical replicates.

activity of MB73/2 *spo0A*- on potato slices. As expected, the mutant strain exhibited comparable ability to reduce the maceration activity of *D. solani*, confirming that Spo0A is not directly involved in the antagonistic interaction. This results further support the assumption that screening the mutants on semi-solid medium yields consistent results when translated to *in vivo* studies.

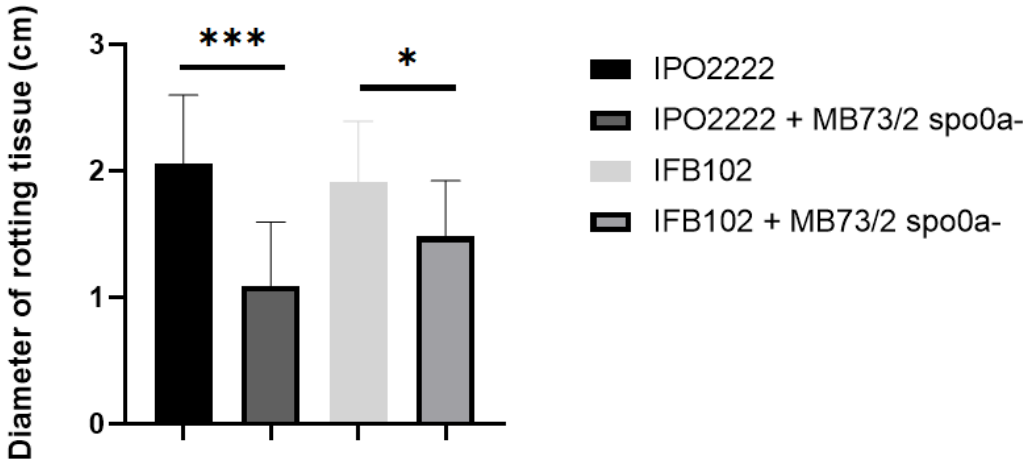

Figure 35- Diameter of rotting tissue (mm) on 1.5cm potato slices inoculated with IPO2222 and IFB102 alone or in co-inoculum with MB73/2 spo0A 96hours after infection. Error bars indicate standard deviation. The experiment was repeated two times with three technical replicates.

These findings align with our previous results where we show that MB73/2 and 168 show high genomic identity along with the formation of an inhibition zone when they interact with *D. solani* on solid and semi-solid medium. Combined results, support the application of *B. subtilis* MB73/2 for biocontrol strategies in agriculture to manage soft-rot disease caused by *D. solani*. However, further research is needed to investigate the efficacy of *B. subtilis* under field conditions. One important consideration is the potential formation of *B. subtilis* biofilms on treated potatoes, as observed when extending the incubation time under laboratory conditions (data not shown). Biofilm formation could have implications for potato health and yield, potentially affecting crop growth and susceptibility to other pathogens or environmental stressors.

9 *Dickeya solani* wild type strain IPO2222 and the environmental strain IFB102 phenotypic and genetic differences

9.1 IFB102 and IPO2222 exhibit different motility and antagonistic interaction with MB73/2

D. solani strains exhibit an exceptionally high level of genome homogeneity, even among strains isolated from soft-rotting plants or from the rhizosphere of healthy plants. This remarkable homogeneity is underscored by ANIb values ranging from 98.55% to 99.93% and ANIm values ranging from 98.71% to 99.92%, as demonstrated in a recent pangenome analysis performed on 22 *D. solani* genomes (Motyka-Pomagruk et al., 2020). Furthermore, in a recent study involving 14 *D. solani* strains, it was found that the genes encoding major virulence determinants such as pectinases, cellulases, and proteases, along with their regulators (KdgR, PecS, PecT, Fis, H-NS, and Fur), exhibited 100% identity. Despite this high level of genomic identity, strains were found to vary significantly in virulence, production of plant cell wall-degrading enzymes, and motility (Golanowska et al., 2018). This discrepancy led us to investigate phenotypic and genotypic differences among the wild type strain IPO2222 and the environmental isolate IFB102.

When it comes to swarming motility *D. solani* IFB102 and IPO2222 exhibit very different phenotype under laboratory conditions. Specifically, swarming of IFB102 show the typical *dendritic* pattern with formation of swarming tendrils departing from the inoculation point, while IPO2222 has unidirectional and irregular swarming, forming a characteristic *vortex* pattern with dense clusters of cells on the agar surface, resulting in a notably moist environment within the plate (Fig. 37). This variability in swarming motility among *D. solani* strains is consistent with previous findings reported by Pomagruk and collegues in 2023. Their study highlighted the considerable diversity observed in swarming behaviour across different strains of *D. solani*, which was partially correlated to the virulence of the different strains.

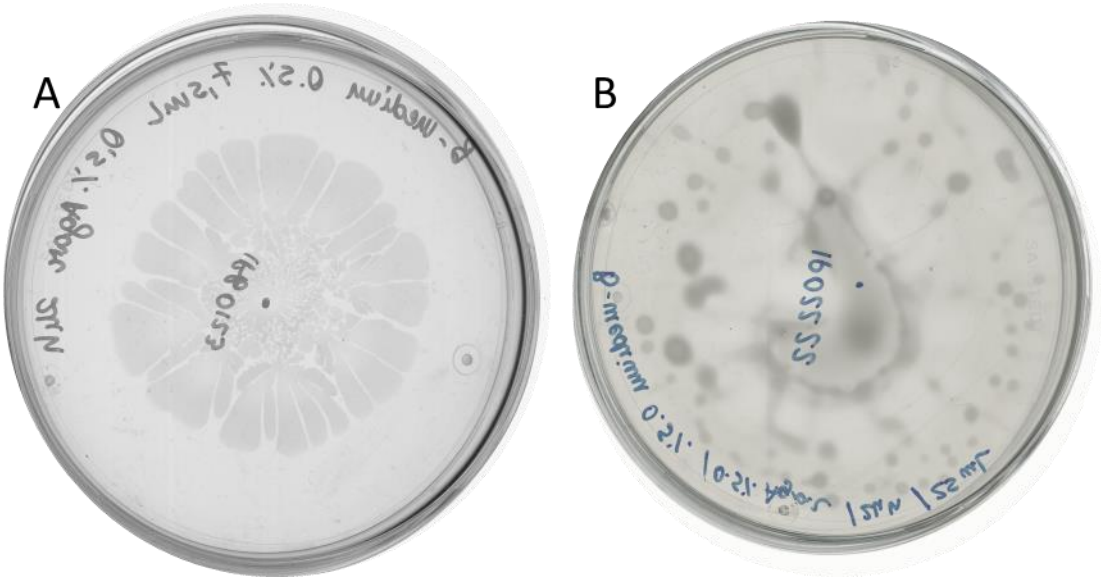

*Figure 37- Swarming of D. solani IFB102 (A) and IPO2222 (B) on 7.5*ml *of 0.5x B-medium with 0.5% of agar 24h upon inoculation*

The distinct swarming phenotypes suggest potential differences in underlying genetic mechanisms governing motility. However, the phenotypic differences extend beyond motility. Upon interaction with MB73/2 on a swarming assay plate, IPO2222 directionally escaped from the advancing *B. subtilis*. However, we could not observe the formation of the inhibition zone between the interacting species (Fig. 38). Instead, *B. subtilis* was able to efficiently colonize the inoculation point of IPO2222, suggesting that the wild type strain of *D. solani* lacks the ability to inhibit *B. subtilis* growth.

9.2 The sRNA ArcZ is not responsible for the lost of bacteria growth inhibition of strain IPO2222

A study by Brual and colleagues in 2023 provides insights into the interactions between *D. solani* strains and other microorganisms, including *B. subtilis*. Their research focused on investigating the inhibitory capabilities of *D. solani* strains against both fungi and bacteria, revealing strong phenotypic differences among the *D. solani* strains. Specifically, strain IPO2222 was found to lack the ability to inhibit *B. subtilis*, consistent with our observations. The authors excluded the involvement of the NRPS and PKS gene clusters in the interaction and, considering the high genomic identity among *D. solani* strains, rather attributed the different phenotype to SNPs (single nucleotide polymorphism). In particular, they identified a SNP (a A at position 90 instead of a G) into the *arcZ* region. ArcZ is a sRNA that works as post-transcriptional regulator. The

authors showed, by knockout and complementation assay, that this mutation at position 2530087 in IPO2222 genome is responsible for the loss of inhibition of *B. subtilis*.

These fundings led us to investigate the sequence of arcZ in the strain IFB102. To do so, we sequenced genomes of strain IFB102 and our version of strain IPO2222. Whole genome sequencing was conducted by Genomed (company). The reads were assembled in 53 contigs. Nine filtered scaffolds generated from the contigs were used to obtain the consensus sequence, using as reference the genome of IPO2222 from GenBank. Subsequently, we used Mauve and megablast to compare the genome of the two strains. Our analysis revealed that our IPO2222 harboured the same mutation reported in the literature at position 2530087, while *arcZ* sequence of strain IFB102 was found to be identical to that of D s0432-1, which served as wild type in Brual's work.

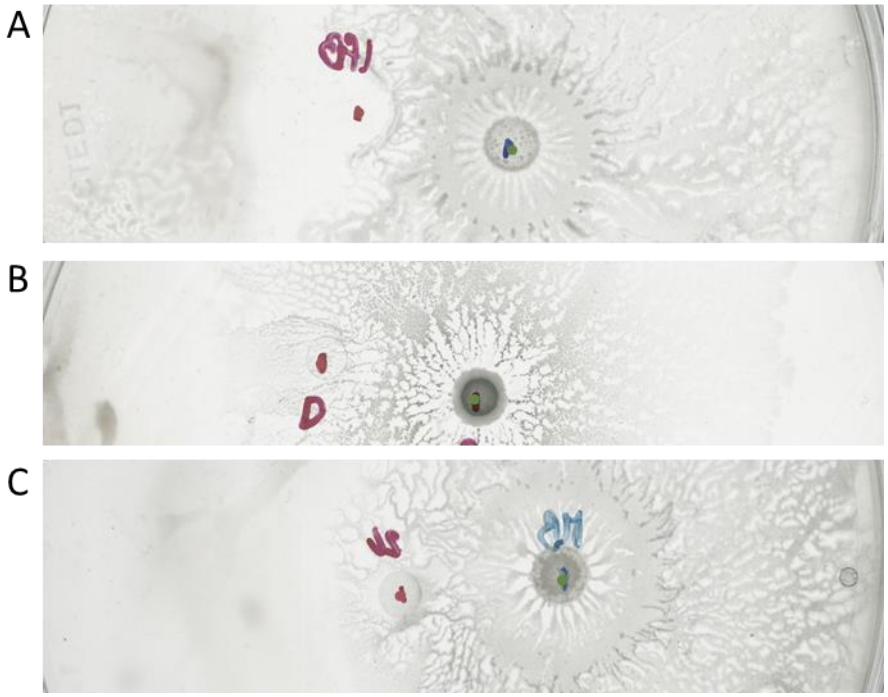

Figure 38 - Interaction on swarming assay between MB73/2 and IFB102 (A), D s0432-1 (B), IPO2222 (C). The inhibition zone is visible only in antagonism with IFB102. Red dots indicate the point of inoculation of D. solani strains. Green dots indicate point of inoculation of MB73/2

Considering the involvement of ArcZ in the interaction with *B. subtilis*, we acquired strain D s0432-1, which contains the wild type version of *arcZ*, and proceeded to evaluate its ability to inhibit *B. subtilis*. However, contrary to our expectations, strain D. s0432-1 exhibited the same phenotype as strain IPO2222. Upon interaction with MB73/2 in a swarming assay, we were unable to detect any inhibition zone and *B. subtilis* was able to colonize the area previously occupied by *D. solani* (inoculation point). This contradictory result can be explained in two ways. Firstly, the *B. subtilis* strain used in Brual's work is

the laboratory strain PY79, whereas we used the environmental strain MB73/2. Since PY79 has not been utilized in our experiments, it is possible that the laboratory strain may react differently to the antimicrobials released by *D. solani* compared to an environmental strain such as MB73/2. Moreover, the inhibition of growth in Bural's work was solely verified in a spot-on-lawn assay where bacteria do not establish any form of motility. On the other hand, our results on motility suggest that IPO2222 and D s0432-1 equally exhibit uncontrolled swarming motility compared to IFB102. Our previous findings lead us to consider that the antagonism between *D. solani* and MB73/2 strongly depends on the ability of the two species to sense each other's presence and react. IPO2222 and D. s0432-1 swarm faster than IFB102. Therefore, it is plausible that they leave the point of inoculation earlier, allowing insufficient time for efficient production and accumulation of the antimicrobial compounds that inhibit *B. subtilis* growth. This early dispersal may result in the absence of an inhibition zone and subsequent colonization of the inoculation point by *B. subtilis*. Since swarming motility is a quorum sensing and coordinated behaviour, it is possible that other genes beyond *arcZ* are responsible for the impaired antagonism.

9.3 Strain IPO2222 and IFB102 exhibit different metabolic response to carbon sources

The variance in virulence observed between strains with impaired or uncontrolled motility is to be expected. Motility, combined with chemiotaxis, plays a crucial role in allowing bacteria to move towards or away from certain stimuli. We showed that the carbon source is important to support swarming motility of *D. solani* and we wanted to investigate whether IPO222 and IFB102 exhibit different motility due to their different metabolic response to D-sugars.

Bacteria were grown in the synthetic medium MMA, following the method established for the close relative *D. dadantii* 3937 (Lamas et al., 2009). Bacteria (starting at OD600 of 0.03) were cultivated in the MMA medium containing different carbon sources (sorbitol, glucose, fructose, sucrose, mannose) for 24h and the endpoint OD600 was measured. As expected, in the carbon-source-free MMA* medium, both strains exhibited similarly low growth rates, indicating a dependence on external carbon sources for growth.

However, it is interesting to note that IFB102 and IPO2222 respond differently to the different carbon sources tested (Fig. 39). While both IPO2222 and IFB102 exhibited impaired growth in the presence of sorbitol, IPO2222 showed highly compromised growth in sucrose-containing medium, suggesting an inability to utilize sucrose effectively as the sole carbon source. Additionally, IFB102 consistently outperformed IPO2222 under most tested conditions, except in the presence of mannose. Particularly significant was the substantially reduced growth of IPO2222 in the presence of glucose ($p<0.05$).

Given the established influence of carbon sources on antimicrobial production in various bacterial species, including *Pseudomonas* (van Rij et al., 2004) and other *Dickeya* species (Liao et al., 2014; Feng et al., 2019), we speculated that the distinct biochemical capabilities of IPO2222 and IFB102 could impact their antimicrobial production against *B. subtilis*. Specifically, the different ability to grow in the presence of glucose might affect the strains capacity to produce sufficient levels of antimicrobials. To delve deeper into these findings, we performed a comparative genomic analysis of IPO2222 and IFB102.

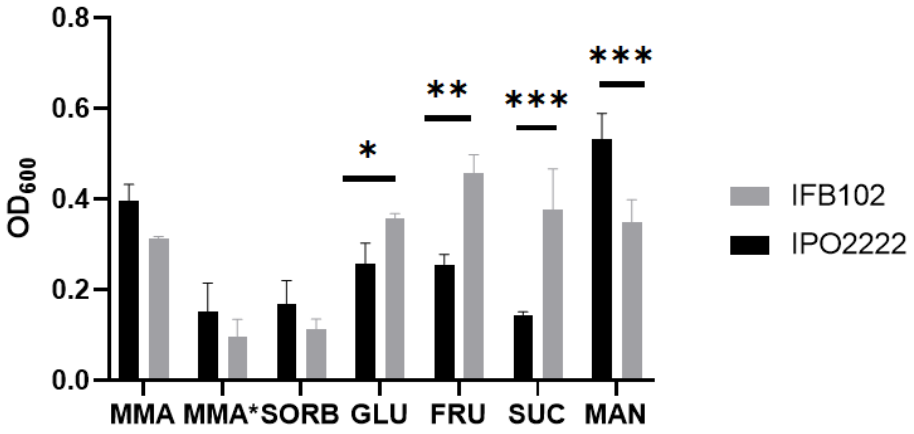

Figure 39 - End point of a 24h growth curve of IFB102 and IPO2222 expressed as OD_{600} in media containing no carbon source (MMA) and different carbon sources: sorbitol (SORB), glucose (GLU), fructose (FRU), succrose (SUC), mannose (MAN). Stars indicate the level of significance ($p<0.05$, $p<0.01$, $p<0.001$). Error bars indicate standard deviation. The experiment was repeated twice with six technical replicates.*

9.4 Comparative genome analysis of *D. solani* IFB102 and IPO2222 revealed only six SNPs

D. solani IFB102 and IPO2222 show differences in motility, metabolism and antagonistic interaction with *B. subtilis* MB73/2. This prompted us to consider the involvement of a transcriptional regulator, rather than a single gene, in the regulatory network.

From the genome comparison we identified only 7 SNPs (Tab. 4) between the two strains, with no insertions, deletions, or duplications found. Among those, a G to T substitution at position 4635450 occurred within a transcriptional regulator belonging to the LysR family. LysR-type transcriptional regulators (LTTRs) currently represent the largest known family of bacterial regulators, comprising over 800 identified members based on their amino acid sequences (Maddocks et al., 2008) LysR-type regulators are known as global transcriptional regulators, capable of functioning as either activators or repressors of single genes and operons. The products of the regulated genes serve various functions, encompassing cell metabolism, quorum sensing, virulence, motility, and toxin production. In *Dickeya* species, a well-known LTTR is encoded by the *pecT* gene. PecT is a repressor of *pel* genes expression, thereby influencing virulence (Herault et al., 2013)

To evaluate the role of the LysR-type regulator in the interaction between *D. solani* and *B. subtilis* MB73/2, we constructed a mutant strain of IFB102 carrying a deletion of this gene. The pUC19-LysR plasmid was constructed using Gibson assembly, incorporating a gentamicin resistance cassette flanked by 500bp sequences upstream and downstream of the LysR gene. This cassette was inserted via restriction digestion with BamHI and KpnI. The resulting plasmid was then amplified in *E. coli* DH5α and subsequently transferred into IFB102 via electroporation.

Table 4 - List of the SNPs between IPO2222 and IFB102. The table reports the position in the IPO2222 genome, the type of substitution and the product of the gene

SNPs	*Position*	*IPO2222*	*IFB102*	*Product*
1	2530087	T	C	ArcZ
2	2621920	C	T	Hypotetical protein kinase
3	3554462	C	A	cithocrome d terminal oxidase subunit 1
4	3674549	A	G	HNH/endonuclease VII fold putative polymorphic toxin
5	4039850	G	A	intergenic region
6	4513052	A	G	Hypotetical protein
7	4635450	C	T	transcriptional regulator - LysR family

The phenotype of the IFB102 Δ*LysR* strain was analysed for swarming motility and its interaction with *B. subtilis* MB73/2. The observed phenotype closely mirrors that of IPO2222, displaying enhanced motility and no inhibition zone when antagonized with *B. subtilis* (Fig. 40).

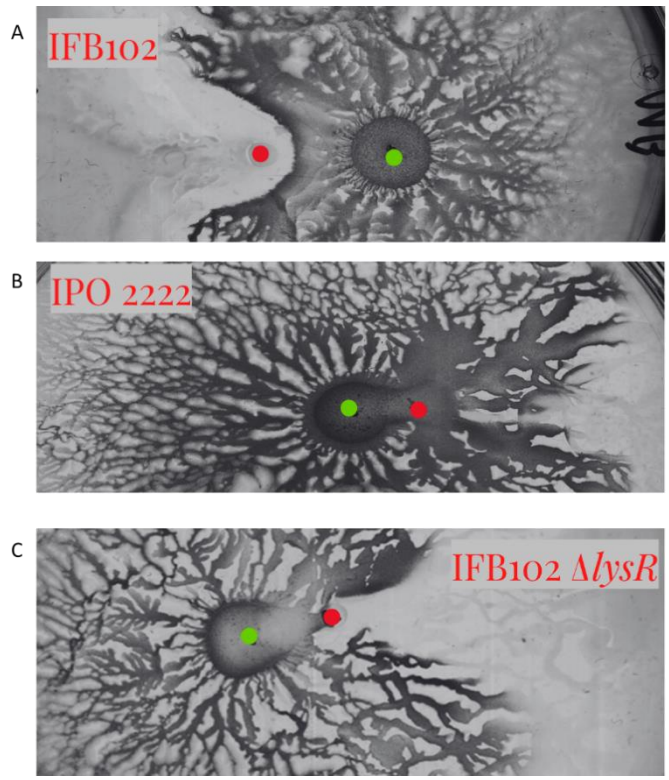

Figure 40 - Swarming interaction between MB73/2 and D. solani IFB102 (A), IPO2222 (B) and IFB102ΔLysR. The inhibition zone is not visible in both the mutant and the laboratory strain. Red dots mark the point of inoculation of D. solani strains and green dots mark the point of inoculation of MB73/2

It is important to note that strain D s0432-1, which was showing the same phenotype of IPO2222 in swarming motility and antagonistic interaction, present the same mutation at position 4635450. Therefore, our result suggests that the LysR-regulator might be involved in the regulation of motility and secretion of antimicrobial compounds. Further studies should address the regulon of this LysR-type transcriptional factor and should focus on elucidating the functional consequences of the G to T substitution with the *LysR* gene.

VI. CONCLUSIONS

The emergence and wide dissemination of *Dickeya solani* in potato production mark a significant shift in the epidemiology of potato blackleg disease. Previously dominated by other *Dickeya* species and *Pectobacterium atrosepticum*, the ascent of *D. solani* over the past two decades has fundamentally altered the landscape of potato cultivation in Europe and beyond. This radical shift can be attributed to the heightened virulence of the pathogen and its broader range of optimal temperatures for disease development.

Despite efforts to mitigate the spread of SRP, including *D. solani*, through stringent regulations and monitoring protocols, the prevalence of SRP continues to pose challenges to potato production, leading to yield reductions, seed rejection and downgrading, additional costs for growers, and post-harvest losses.

Traditional methods, including chemical and physical treatments, have proven inadequate for the effective management of infection caused by *D. solani*. Aligned with the European Commission's directive to reduce pesticide use in agriculture, alternative strategies involving microbial control agents (MCAs) have garnered attention for managing of *D. solani* infections.

At present, *Bacillus* species are by far the most widely used bacteria in bioformulations due to their ability to form endospores that can resist to biotic and abiotic stress, secrete a wide range of antimicrobial compounds and enhance plant growth and soil health.

In this study we evaluated the ability of the environmental strain *B. subtilis* MB73/2 to limit the growth and the virulence of *D. solani* IFB102. Our investigation into bacterial multi-species interactions employed a comprehensive approach, integrating conventional planktonic cultures with semi-solid agar assays to better represent the natural environment interactions.

Our attempt to induce swarming motility under laboratory conditions, yielded significant insights into the pivotal role played by medium type, volume, and humidity levels in supporting swarming motility. These factors emerge as critical determinants influencing the accumulation and detection of AHLs signalling molecules within the colony proximity, thereby facilitating or limiting the expression of swarming motility.

Furthermore, we observed a strong interplay between glucose concentration, carbon catabolite repression, AHLs production, and swarming motility, underscoring the intricate

regulatory mechanisms bacteria employ to adapt their behaviour to environmental changes and optimize resource utilization. This shift in focus from motility to metabolic efficiency is accompanied by a decrease in AHLs production. It's reasonable to speculate that the reduced presence of quorum sensing signalling molecules in the medium under glucose-rich conditions inhibits the activation of swarming behaviour. Thus, we registered a strong interplay between glucose concentration, carbon catabolite repression, AHLs production, and swarming motility.

Combined results highlight the intricate regulatory mechanisms bacteria employ to adapt their behaviour to environmental changes and optimize resource utilization. In addition, it allowed us to establish a robust laboratory protocol for the induction of swarming motility of *D. solani* under controlled laboratory settings.

We aimed to investigate the interaction between *D. solani* and *B. subtilis* when bacteria interact as cell-collectives and establish social interactions, such as during swarming motility. Our investigation revealed a complex prey-predator antagonism rather than a simple avoidance mechanism. The formation of an inhibition zone between the interacting bacteria, a sharp front that *B. subtilis* is not capable of penetrating and the coordinated and directional escaping of *D. solani*, further support this hypothesis.

Moreover, the inhibition zone can be more properly defined as interaction zone where both bacteria secrete antimicrobial compounds upon sensing each other presence which result in: (i) the formation of *B. subtilis* multi-layered wrinkled biofilm at the front of the interaction and (ii) the coordinated and directional escape of *D. solani* from the point of inoculation.

It is important to note that the CFS of *B. subtilis* MB73/2 resulted completely ineffective in reducing the growth or altering motility of *D. solani* these findings, combined with the lack of significant growth reduction when bacteria were co-cultured in liquid medium, strongly support our hypothesis of a sensing-response mechanism governing the secretion of antimicrobial compounds. These findings, combined with the lack of significant growth reduction when bacteria were co-cultured in liquid medium, strongly support our hypothesis of a sensing-response mechanism governing the secretion of antimicrobial compounds.

Furthermore, the observed heterogeneity and segregation of cells in the co-inoculum swarming pattern confirm the ability of bacteria to recognize members of the species,

engage in antagonistic interaction, and swarm independently from their competitor. The delayed formation of the inhibition zone, visible only after 48 hours during the spot-on-lawn assay, further supports our hypothesis, suggesting that antimicrobials are secreted upon sensing the presence of antagonism and accumulate over time.

B. subtilis produce a wide range of molecules with known antimicrobial proprieties, including the biosurfactant surfactin. However, our investigations unveiled that surfactin does not directly mediate the inhibition of *Dickeya solani* growth. Instead, we discovered that surfactin plays a crucial role in promoting the swarming behaviour of the pathogen, while also being essential for orchestrating its directional escape. Therefore, we speculated that surfactin facilitates the diffusion of the antimicrobials produced by *B. subtilis* in the medium while enhancing membrane permeability to AHLs thereby fostering a faster and coordinated escaping of *D. solani*.

In addition, the master regulator Spo0A is well known to play a crucial role in the production of antimicrobial compounds in *B. subtilis*. Our results on the MB73/2 deletion mutant led us to exclude its involvement in the secretion of antimicrobial against *D. solani*. Moreover, the extensive study on deletion mutants from the BKE library, based on the list of genes involved in the synthesis of antimicrobials, suggests that none of the known antimicrobial compound produced by *B. subtilis* is responsible of the antagonism against *D. solani*.

It is important to note that our approach to understanding the molecular mechanism of interaction relied on constructing mutants through random mutagenesis.. However, the use of the chemical mutagen MNNG induced several genetic variations that posed significant challenges for downstream analysis. At the same time, results from transposon mutagenesis turned out to be inclusive, prompting us to consider the secretion of these compounds a multi-factorial event that cannot be attributed to a single gene effect.

Furthermore, we observed phenotypic variation in terms of swarming motility and interaction with *B. subtilis* between the wild type strain of *D. solani* IPO2222 and the environmental isolate IFB102. NGS data analysis identified only 7 SNPs among the two genomes, with the deletion of the LysR-type regulator providing compelling evidence of its involvement in the secretion of antimicrobial compounds against *B. subtilis*, resulting in a loss of function in the deletion mutant.

Despite the limited results on the molecular mechanism governing the antagonism between *D. solani* and *B. subtilis*, in vivo studies on potato slices further support the effectiveness of *B. subtilis* in limiting pathogen growth and soft rot disease development, presenting *B. subtilis* MB73/2 as a valid candidate for field testing. Further research should be dedicated to investigating the application of *B. subtilis* under field conditions and further elucidating the specific mechanisms underlying the observed antagonism between these two species.

Part of results presented in this work have been published in the following publication: Gatta R, Wiese A, Iwanicki A, Obuchowski M. Influence of glucose on swarming and quorum sensing of *Dickeya solani*. *PLoS One*. 2022 17:e0263124. doi: 10.1371/journal.pone.0263124.

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