A Bioaugmentation Approach to Pesticide Bioremediation: Pesticide Degradation and Crop Protection

MASTER THESIS, SUSTAINABLE BIOTECHNOLOGY SECTION

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ABSTRACT

Dicamba is a benzoic acid herbicide that is used to control annual and perennial broadleaf weeds in grain crops and grasslands. Dicamba does not bind to soil particles and is highly soluble in water, which grants it high mobility in the soil with the possibility of contaminating groundwater. Also, it tends to spread from treated fields into neighboring fields, causing damage.

The aim of this study was to find organisms that are capable of degrading Dicamba, and at the same time, through a collaboration with Copenhagen University, see if those organisms are able to act as biological control agents against the fungal plant pathogen *Fusarium culmorum*.

A solid medium was devised that selects for pesticide degrading organisms, along with degradation experiments in liquid medium, which led to finding 4 bacterial species that can degrade the pesticide Dicamba.

Since the main purpose is to find the organisms that possess the dual function of pesticide degrader and biological control agent, those organisms that proved to be able to degrade the pesticide were used in sand assays with wheat seedlings, along with *Fusarium culmorum*, by coating the seeds first with the fungal pathogen and then the possible biocontrol agents. The setups were incubated for 2 weeks and then assessed depending on the severity of the symptoms caused by the *Fusarium*. At the same time, different concentrations of Dicamba were applied on wheat seed to determine if Dicamba could be included in the Fusarium sand assays, with the 6.25 mg/L concentration being chosen as a suitable working concentration.

Out of the organisms tested, none exhibited a dual function, while there are some organisms that were able to degrade Dicamba, those were not able to significantly decrease the symptoms caused by *Fusarium culmorum* on the wheat seedlings.

1. INTRODUCTION

1.1. Pesticides

A pesticide can be defined as any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating pests. Any substance or mixture of substances intended for use as a plant regulator, defoliant, or desiccant can also be included in the definition. Pesticides are biologically active compounds, and, in time, the mechanism has shifted from directly killing the pests to interfering with metabolic processes. As a result, they could interfere with metabolic processes also in humans and other living organisms. Over a period of almost forty years, concern about pesticide use and the side effects on man and the environment have prompted safety requirements governing use (RAGSDALE 1999).

As a result, pesticides present a wide range of benefits, but also risks associated with their use and overuse (Tab. 1) (Council 1993).

Table 1. Benefits and risks associated with the use of pesticides (Council 1993).

Benefits of pesticide utilization	Associated risks
Crop protection	May present high toxicity to humans
Food and material preservation	High impact on the environment
Disease control	Prone to bioaccumulation

The main function of pesticides is to ensure crop protection. It is estimated that the annual worldwide crop loss from plant diseases, insects and weeds is about 220 billion dollars. Thus, the need for pesticides is present (Oerke and Dehne 2004).

1.2. History of the use of pesticides

The beginning of agriculture can be traced back to around 10 000 years ago, being implemented in the Fertile Crescent of Mesopotamia (parts of present day Iraq, Turkey, Syria and Jordan) (Kislev, Weiss et al. 2004). Initially, edible seeds were gathered by a population of hunter/gatherers, which evolved into the cultivation of wheat, peas, lentils and barley. In a similar pattern, rice was cultivated in China and in the Sahel region of Africa about 7 500 years ago. On the American continent, corn, squash and sunflower were domesticated (Bocquet-Appel 2011).

It soon became clear that if the crops were to suffer from different pests and diseases, it would cause a great loss in feed, which would also involve famine for the population. As a result, the need for compounds that could inhibit or potentially kill pests was increasing (Pimentel 2002).

Regarding the development of modern pesticides, Costa marks the period between 1935 and 1955 as the one in which DDT and other chlorinated hydrocarbon pesticides were developed. Although DDT was first synthetized by Zeidler in 1874, only in 1939 was it found that DDT acts as a contact poison on flies, mosquitoes and other various insects. One of the most successful uses of DDT has been in the malaria eradication programs. As an example, in 1944 in Italy, there were 50-60 cases of malaria per 1000 inhabitants. After the DDT spraying program that was started in 1945, the incidence dropped to 0 by 1949 (Costa 1987).

In 1962, the book Silent Spring by Rachel Carson was published, which mentions the effects DDT has on the environment and on bird reproduction. The book, along with the discussions and awareness raised would eventually lead to a ban on DDT both in Sweden (1970) and the USA (1973) (Costa 1987).

1.3. Types of pesticides

The classification of pesticides is mainly based on their chemical nature (Tab. 2), application requirement (agriculture, public health, domestic) and target organism or targeted use (insecticide, herbicide, fungicide) (Jayaraj, Megha et al. 2016).

Table 2. Classification of pesticides based on their chemical nature (Jayaraj, Megha et al. 2016) (modified).

Chemical Group	Chemical Name
Organochlorides	DDT, Lindane, Chlordane, Endosulfan, Isodrin, Isobenzan, BHC
Organophosphates	Malathion, Dimethoate, Trichlorfan, Demetox, Dichlorovas, Abate, Phorate, Dimefox, Mipafox
Carbamates	Carbaryl, Prupoxur, Pebulate, Monilate, Naban, Zineb, Maneb
Pyrethroids	Allethrin, Bonthrin, Dimethrin, Tetramethrin

Phenyl amides	Barban, Carbetamide, Solan, Karsil, Trifluralin, Benefin
Phenoxyalkonates	Dichloroprop, Mecoprop, Erbin, Sesone
Benzoic acid	Dicamba, Simazine, Ametryn

Organochlorides:

Organochlorides (OC) are a group of chlorinated compounds that belong to the class of persistent organic pollutants, as they present high persistence in the environment.

Dichlorodiphenyltrichloroethane (DDT) is the most known organochloride pesticide. DDT is notorious for raising numerous human health and environmental issues in the past. Even though its toxicity to humans is low, which led to its widespread use, it is slowly biodegraded, as a result it persists in the environment. The persistence of DDT in the environment leads to bioaccumulation in the tissues of living organisms. DDT is stored in all tissues, but the highest levels of accumulation can be found in fat deposits (Turusov, Rakitsky et al. 2002).

Even though it was banned between the years of 1970-1973, DDT and its metabolites can still be found in samples taken from various fish and birds analyzed (Turusov, Rakitsky et al. 2002).

According to Gupta, in 2004 approximately 40% of pesticides used worldwide belonged to the organochlorides group (Gupta 2004). This can be attributed to their low cost, but also to the benefits they provided when utilized for the control of malaria and typhus (Aktar, Sengupta et al. 2009). Due to the aforementioned low cost, pesticides belonging to the organochlorides group are among the most widely used in developing countries (Gupta 2004).

Organophosphates:

Organophosphates (OP) are esters of phosphoric acid. The function of the OP pesticides is exerted through irreversible inactivation of acetylcholinesterase, an enzyme essential for nerve function in humans, insects and other animals. Organophosphates are degraded rapidly by exposure to light, air and soil, however small amounts in food and drinking water have been reported (Jayaraj, Megha et al. 2016).

Initially, organophosphates were promoted as a more environmentally-friendly alternative to organochlorides (Jaga and Dharmani 2003), but because of their extensive use and easy accessibility, they represent the major cause of acute pesticide poisonings and deaths around the world (Karami-Mohajeri, Hadian et al. 2013).

Malathion (Fig. 1) is an organophosphorus pesticide with wide utilization in agriculture and homes, as protection from various pests. It is a cholinesterase inhibitor that is used as a topical pediculicide. Malathion attacks the nervous system of lice through inhibition of cholinesterase activity, therefore allowing acetylcholine to accumulate at cholinergic synapses, and enhancing cholinergic receptor stimulation, which in turn leads to the death of the lice. Malathion is also utilized to control mosquitoes and other flying insects (Kim, Chen et al. 2018).

Malathion present in the soil will not migrate into air and water surfaces, and it is expected to move slowly or not at all through soil (Kim, Chen et al. 2018).

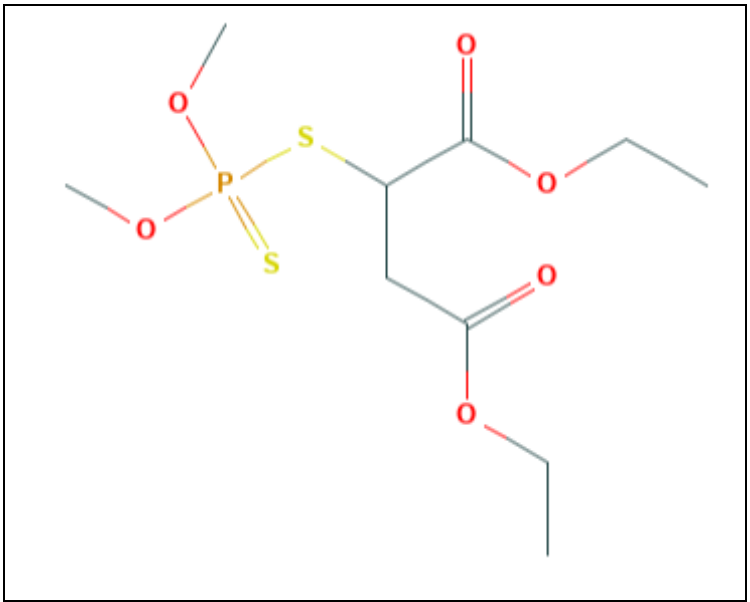

Figure 1. Chemical structure of Malathion (Kim, Chen et al. 2018).

Research shows that malathion can lead to a variety of syndromes and effects that include human breast carcinoma, genetic damage, disrupted hormone activity and hepatotoxicity (Ibrahim, Karam et al. 2014).

Dimethoate is a broad range organophosphorus insecticide, which was introduced in 1956 and has been produced in many countries ever since, mainly for use in agriculture, but also for the control of household insects. Similar to most organophosphates, its mechanism of action is acetylcholinesterase inhibition, which leads to nerve exhaustion and ultimately death. It is one of the most important pesticides in the Kashmir valley, because of its abundant use (Qayoom, Shah et al. 2016).

Dimethoate is a monocarboxylic acid amide that has a role as an agrochemical, an acaricide, an insecticide and a xenobiotic and environmental contaminant (Fig. 2).

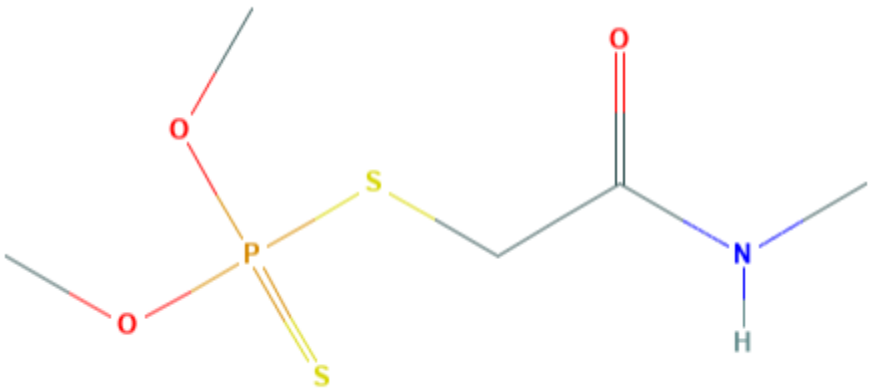

Figure 2. Chemical structure of Dimethoate (Kim, Chen et al. 2018).

Carbamates:

Carbamates are derivates of carbamic acid. The function of carbamates is exerted through the reversible inactivation of acetylcholinesterase, and they are broken down in the environment within weeks or months (Goel and Aggarwal 2007).

Pyrethroides:

Pyrethroides are compounds isolated from the flowers of pyrethrums (*Chrysanthemum coccineum* and *Chrusanthemum cinerariaefolium*). Their mechanism of action is through targeting of the sodium channels, which leads to paralysis of the organism. Pyrethroides have a low level of mammalian toxicity and they are biodegraded rapidly (Jayaraj, Megha et al. 2016).

Benzoic acid:

Benzoic acid herbicides include dicamba, chlorambin, bromoxynil, dichlobenil and naptalam.

Dicamba (Fig. 3) is an auxin synthetic herbicide that is used to control more than 200 kinds of broadleaf weeds. It is chemically stable, but does not persist in soil or water, which indicates that its degradation in the environment is biologically mediated (Yao, Yu et al. 2016).

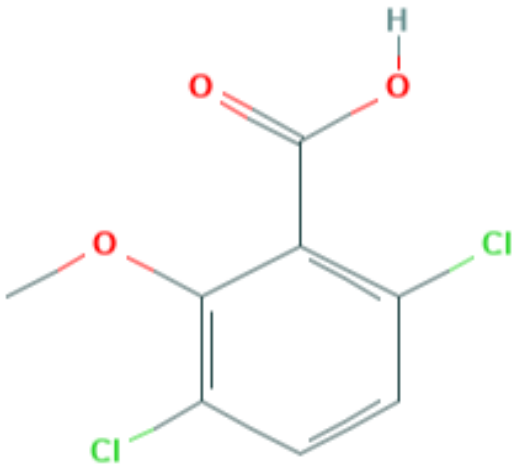

Figure 3. Chemical structure of Dicamba (Kim, Chen et al. 2018).

1.4. Environmental impact of pesticides

Pesticides can contaminate water, soil, turf and other types of vegetation. While their effects lie in killing insects and various weeds, pesticides can affect birds, fish, beneficial insects and valuable plants (Aktar, Sengupta et al. 2009).

Water contamination can be separated into surface water contamination and ground water contamination. Regarding surface water contamination, it is caused by runoff from treated plants and soil. According to Kole et al, in 2001, in a study conducted on river basins in the United States by the U.S. Geological Survey, more than 90% of water and fish samples from all streams contained at least one type of pesticide (Kole, Banerjee et al. 2001).

Ground water contamination seems to be a global issue. In studies conducted in the United States, pesticides have been detected in ground waters of over 43 states, and in India, sampling of drinking water supplies resulted in 58% testing positive for organochloride pesticides at higher than accepted dosages for humans (Aktar, Sengupta et al. 2009).

Soil contamination is mainly determined by certain characteristics of pesticides, such as hydrophobicity and persistence. The pesticides responsible for this area of contamination are generally organochlorides, such as DDT and lindane, and even though they have been banned in agriculture for a considerable amount of time, residues can still be found. (Andreu and Pico 2004)

1.5. Pesticide degradation

Bioremediation can be described as a process that utilizes microorganisms or their products in order to treat polluted sites, to restore them to their original condition (Ying 2018).

Bioremediation can be categorized as either in situ or ex situ. In situ bioremediation can be described as a process where a contaminant is biologically degraded under natural conditions to carbon dioxide and water or an attenuated transformation product. In the case of ex situ bioremediation, the contaminated soil has to be excavated and moved to another location where the treatment is applied, which increases the costs involved. Bioremediation processes include bio attenuation, which depends on the natural processes of degradation, biostimulation, which is achieved by addition of nutrients and bioaugmentation, where organisms able to degrade the pollutant (for example a pesticide) are added to the site (Ying 2018).

Pesticides are not approved for use unless they are demonstrated to not be persistent in the environment beyond their intended activity. Even though that is the case, pesticides have been found in numerous natural environments in small quantities (nanograms/liter to micrograms/liter). A warning sign is that among the pesticides detected, almost half are pesticides which have been out of use for a considerable amount of time, while 20% are represented by stable transformation products. Degradation of pesticides involves abiotic processes, such as chemical and photochemical degradation, and biotic processes, mediated by microorganisms and sometimes superior organisms, such as plants (Fig. 4) (Fenner, Canonica et al. 2013).

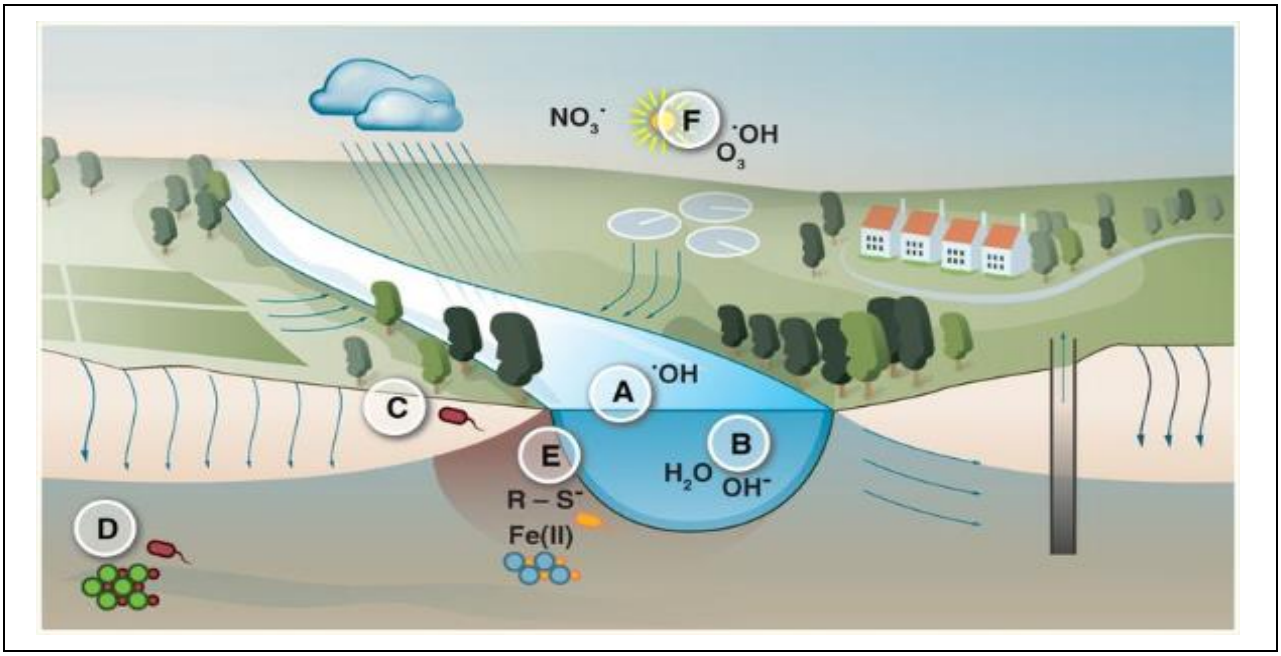

Figure 4. Abiotic and biotic reactions taking place in different segments of an ecosystem.
A-Sunlit surface water; B-Bulk water body; C-Vadose zone; D-Groundwater, oxic; E-Groundwater, anoxic; F-Troposphere (Fenner, Canonica et al. 2013).

There are numerous factors involved in the degradation of pesticides. For example, photochemical degradation can only take place in compartments exposed to sunlight (surface of lakes and rivers, submillimeter layers of soil). Redox gradients present in soils and sediments also play an important role (Fenner, Canonica et al. 2013).

1.5.1. Abiotic degradation

Abiotic transformation can occur in the presence of light (sunlight-mediated) or through dark abiotic transformations. Phototransformation can have high impact in pesticide degradation. Phototransformation can be parted in 2 categories: direct photolysis, meaning that the photons are absorbed by the contaminant itself, and indirect photolysis, meaning that the reactive species are formed through photon absorption by other water constituents (Fig. 5). Due to the spectrum of sunlight being very different from the electronic absorption spectrum of most pesticides, only few pesticides can be transformed through direct phototransformation. Since many active light absorbers are present in surface waters (dissolved natural organic matter, molecular oxygen, superoxide radical anions), indirect phototransformation is more likely to occur (Fenner, Canonica et al. 2013).

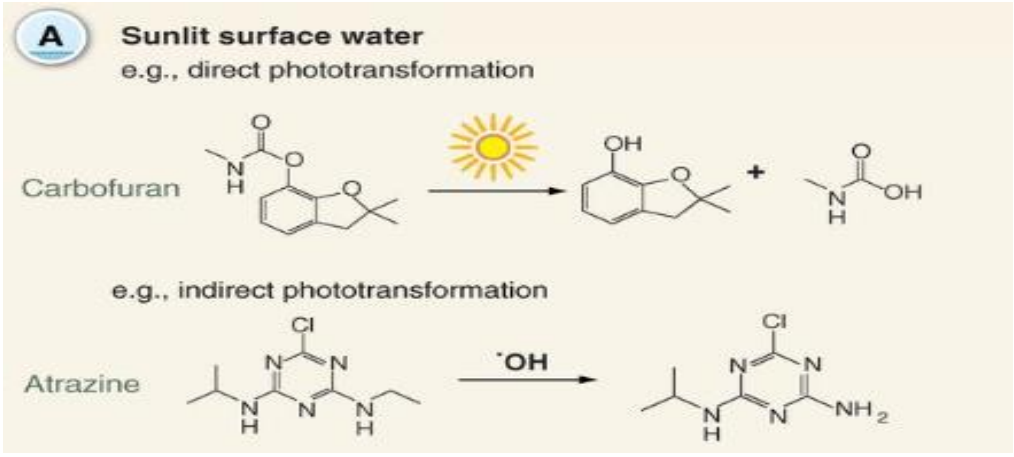

Figure 5. Direct and indirect phototransformation (Fenner, Canonica et al. 2013).

Regarding dark abiotic reactions, their efficiency can differ greatly depending on the present functional group. As a result, compounds that do not possess suitable reactive groups are recalcitrant to chemical transformation. Dark abiotic transformation in aqueous solution has been established for

organophosphates, carboxylic acid esters and carbonates, but in order for it to compete with microorganism degradation, certain conditions such as high pH must be present (Fig. 6).

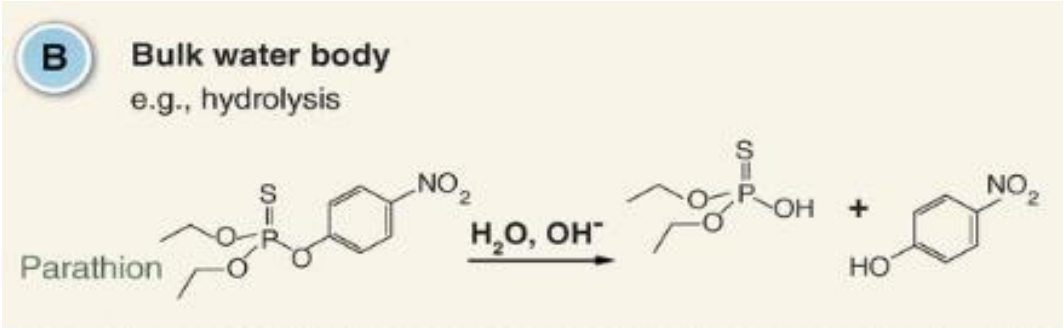

Figure 6. Dark abiotic transformation of the organophosphate pesticide Parathion (Fenner, Canonica et al. 2013).

1.5.2. Biotic degradation

Biodegradation is regarded as the most efficient route of pesticide degradation. While eukaryotes tend to transform the pesticides for detoxification or fortuitous metabolism using broad-spectrum enzymes, prokaryotes metabolize them for assimilation as nutrients and energy. Due to the high evolution and mutation rate, enzymes capable of hydrolyzing pesticides are more likely to be present, while horizontal genetic material transfer ensures spreading of the biodegradation pathways (Copley 2009).

While there are reactions than can occur both biotically and abiotically, some biotical reactions have no counterpart in the abiotic environment due to enzyme requirements. One such case is for example the Carbon-Phosphorus bond (C-P bond) present in glyphosate (Fig. 7), which is resistant to abiotic conditions. An enzyme, C-P lyase, found in microorganisms, is responsible for degradation of glyphosate (Fenner, Canonica et al. 2013).

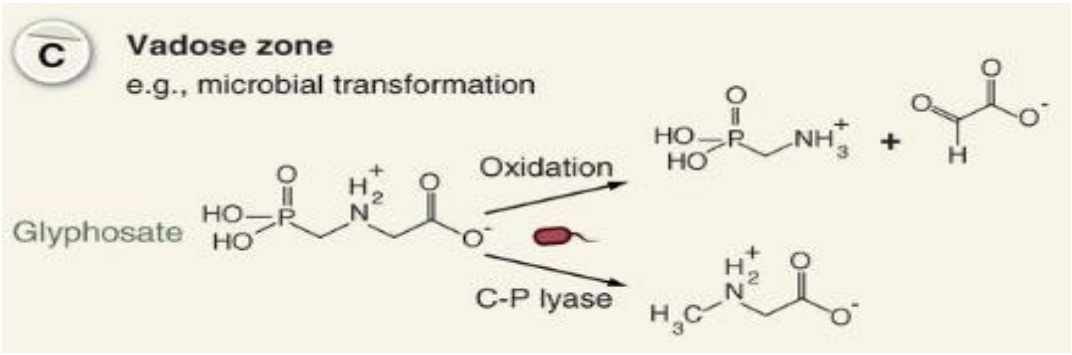

Figure 7. Microbial transformation of glyphosate (Fenner, Canonica et al. 2013).

An example of reaction that can occur both biotically and abiotically is the degradation of Atrazine in groundwater (oxic environment) (Fig. 8). According to Wackett LP et al in a study conducted in 2002, while it was first believed that the degradation of Atrazine can be attributed to abiotic conditions through hydrolytic dichlorination, it appears that bacteria possessing atrazine-dechlorinating enzymes were present, and had a higher degradation rate than the abiotic conditions (Wackett, Sadowsky et al. 2002).

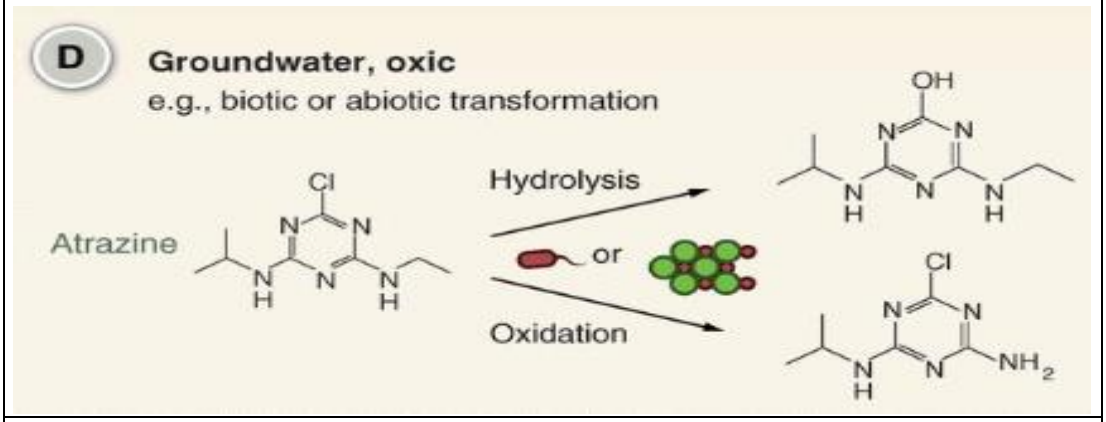

Figure 8. Biotic and abiotic degradation of Atrazine (Fenner, Canonica et al. 2013).

1.6. Pesticides: bioaugmentation and biostimulation

Bioaugmentation

Bioaugmentation is a method of engineered bioremediation and is based on the inoculation of given environments with microorganisms that possess certain capabilities. Bioaugmentation can most efficiently be applied in sites where the number of microorganisms able to degrade the compounds of

interest is insufficient, or the present microorganisms do not possess the necessary pathways for metabolizing the compounds of interest. The inoculation can be performed with either single strains of bacteria/fungi or consortia (Mrozik and Piotrowska-Seget 2010).

Depending on the origin of the inoculants, three methods of bioaugmentation can be distinguished: autochthonous, allochthonous and gene bioaugmentation. In the autochthonous bioaugmentation, microorganisms are isolated from the contaminated environments, enriched, and then reinoculated into the same environment. In the allochthonous bioaugmentation, the microorganisms are isolated from another site and then inoculated into the polluted site of interest (Semrany, Favier et al. 2012). The last method, gene bioaugmentation, utilizes Genetically Engineered Microorganisms (GEMs) that present genes encoding the enzymes responsible for the degradation of the desired compounds (Zhang, Wang et al. 2012).

In 2014, Wang et al have succeeded the bioaugmentation of soil contaminated with Methyl Parathion through inoculation with a strain of *Pseudomonas sp.* They have observed that in both the *Pseudomonas* inoculated soil and non-inoculated soil the degradation of Methyl Parathion was almost complete, but the degradation rate in the bioaugmented soil was higher (13 days to almost complete degradation, compared to 20 days for the non-inoculated soil). The control sample, which was represented by sterile inoculated soil, presented a degradation of 37.2% of total Methyl Parathion, which shows the importance of the microorganisms for degradation of this organophosphorus pesticide (Wang, Chi et al. 2014).

Despite being a very viable approach to bioremediation, bioaugmentation can still have unpredictable outcomes due to the presence of many biotic and abiotic factors. While factors such as pH, temperature, initial pesticide concentration and the presence of additional carbon sources play a major role, it seems that the most important factor is the ability of the inoculum to survive in the polluted environment. If the inoculum survives, other factors might interfere with the proper development and degradation abilities of the culture, such as the loss of the degradative abilities and the inhibition of their growth due to accumulation of toxic intermediates that are formed through degradation of the parent compound (Cycoń, Mrozik et al. 2017).

Biostimulation

Biostimulation is a term used to describe the addition of either electron donors, electron acceptors or nutrients in order to stimulate indigenous microbial populations (Scow and Hicks 2005).

According to Ren et al and Rubio-Bellido et al, when high quantities of nitrogen, carbohydrates and phosphorus are added to soils, in the form of organic amendments, both growth and microbial activity are stimulated, as the addition provides many benefits, such as increased soil aggregation, reduced soil bulk density and improved ion exchange capacity. At the same time, organic amendments contain

microorganisms as well, which might participate in pollutant degradation together with the microorganisms already present in the soil (Rubio-Bellido, Madrid et al. 2015; Ren, Chen et al. 2017).

1.7. The crop protection function of bacteria

Many bacterial genera and species are able to degrade various types of pesticides. The most commonly studied bacterial genera for pesticide degradation are *Pseudomonas*, *Bacillus*, *Flavobacterium* and *Klebsiella* (Tab. 3) (Huang, Xiao et al. 2018).

Table 3. Common pesticide degraders and the pesticides degraded (Huang, Xiao et al. 2018) (modified).

Pseudomonas sp	Aldrin, Chlorpyrifos, DDT, Endosulfan, Parathion
Bacillus sp	Chlorpyrifos, DDT, Glyphosate, Methyl Parathion, Parathion
Flavobacterium sp	Diazinon, Glyphosate, Methyl Parathion, Parathion

During a study conducted by Kabra, Ji et al in 2014, that was aimed at the ability of the microalga *Chlamydomonas* to degrade Atrazine, multiple bacterial species were found, mainly *Pseudomonas sp*, *Klebsiella pneumoniae* and *Bacillus subtilis* (Kabra, Ji et al. 2014).

According to Radhakrishan et al, among pesticide degraders, some bacterial species (ex. *Bacillus, Klebsiella*) can provide beneficial effects to plants in the form of increased growth and prevention of pathogen infections (Radhakrishnan, Hashem et al. 2017).

Bacillus sp exhibit a bio-fertilizer effect on crop plants (Tab. 4), as well as crop protection function against various pests, bacterial diseases, fungal diseases, drought and increased salinity (Radhakrishnan, Hashem et al. 2017).

Table 4. Bio-fertilizer effects of various *Bacillus* species on crop plants (Radhakrishnan, Hashem et al. 2017) (modified).

Bacillus species	Plant growth promotion
B. insolitus, B. subtilis, B. methylotrophicus	Increase the length and biomass of shoot, roots and leaves.
B. megaterium, B. subtilis	Enhance fruits and grains yield

B. pumilus, B. megaterium	Solubilize the phosphorus and nitrogen in soil and increase their transport to roots
B. subtilis, B. methylotrophicus	Synthesis of plant growth hormones that trigger plant growth
B. subtilis, B. mojavensis	Secrete ACC deaminase to inhibit plant senescence
B. megaterium, B. methylotrophicus	Enhance the endogenous proteins, amino acids, sugars, photosynthetic pigments and minerals

As a result, certain species of both *Klebsiella* and *Bacillus* can be categorized as Plant-Growth Promoting Bacteria (PGPB). PGPB promote plant growth while also inducing systemic resistance.

In 2018, Liu et al tested the ability of *Klebsiella pneumoniae* to induce systemic resistance in soybean against *Heterodera glycines*. Since a very important factor in a nematode population is the number of females, as it affects the population size, the number of females was quantified. Using *Klebsiella pneumoniae* strain SnebYK, the number of female nematodes was reduced by 58.56% compared to the control sample, and the total population of nematodes that colonize the soybean roots decreased by 47.32% (Liu, Chen et al. 2018).

Through the application of a Biological Control Agent (BCA) to a crop, the obtained effect can be close to or equivalent to the one achieved by application of a fungicide.

In 2001, Alexander and Stewart used *Phytophthora cactorum* infected apple with application of either fungicide or BCA. While the level of disease suppression obtained in the presence of the fungicide was 100%, application of different BCAs resulted in levels of disease suppression between 79%-98% (Alexander and Stewart 2001).

1.8. Designing a Mixed Consortia for pesticide degradation

A major challenge in pesticide degradation is the microorganisms' resilience to inhibitory compounds, such as the pesticides themselves and the transformation products occurring from both biotic and abiotic degradation. Using a mixed consortia is a viable alternative approach, since it provides various advantages over pure cultures (Varrone, Floriotis et al. 2017).

For example, mixed cultures are able to degrade complex substrates, with different degrees of impurities, while also exhibiting higher resistance to inhibiting compounds (Varrone, Heggeset et al. 2015). Aside from being more robust, mixed cultures possess a complementary metabolism, being able to utilize different carbon sources, which is of great importance in in-situ bioremediation of soil, since

there is a strong possibility that instead of only one pesticide, a mixture of different pesticides might be present (Krishna and Philip 2008) (Varrone, Heggeset et al. 2015).

Considering the strong benefits of a mixed culture, it can also be designed through a rather simple method, by mixing the available isolated strains together, in different combinations and numbers, in order to determine the most efficient pesticide degrader for the target pesticide (Fuentes, Colin et al. 2016).

Through this approach, microorganisms that will be included in the mixed consortium can be first evaluated based on their specific abilities and their importance in the culture, such as the biological control function some microorganisms can exert on plants, which includes enhanced growth, protection from certain insects, fungal pathogens and nematodes (Liu, Chen et al. 2018; Mostafa, Khalil et al. 2018).

1.9. Interactions between Biological Control Agents and plant pathogens

Biological control represents the suppression of populations of plant pathogens by living organisms (Köhl, Kolnaar et al. 2019).

Microbial Biological Control Agents (BCA) protect plants from damage through different modes of action, that can be direct or indirect. As direct modes of action, the BCAs can interact with the pathogens by hyperparasitism or antibiosis, while indirect modes of action are obtained through competition for nutrients and space, and through induced resistance and priming in plant tissues (Köhl, Kolnaar et al. 2019).

1.9.1. Direct interactions with plant pathogens

Hyperparasitism

While parasitism is the direct competitive interaction between two organisms in which one of them is obtaining nutrients from the other, hyperparasitism happens when the host is also a parasite. This type of interaction can mainly be observed in fungi (Köhl, Kolnaar et al. 2019).

In the case of biotrophic mycoparasitism, the hyperparasite depends solely on the host fungus for nutrients. As a result, commercial application of these hyperparasites might prove difficult, since it would require living host mycelium as substrate. In the case of necrotrophic mycoparasitism, the hyperparasite gains nutrients from dead host cells but also from other types of organic matter, which would allow for mass production since they do not require the presence of living host mycelium (Köhl, Kolnaar et al. 2019).

Many antagonistic fungal species that engage in hyperparasitism have been found. In 1995, Jeffries reported 30 hyperparasitic species against *Rhuzoctonia solani*, and in 2017, Zheng et al found as well approximately 30 fungal species that show hyperparasitism against rust pathogens (Jeffries 1995; Zheng, Zhao et al. 2017).

Antibiosis through antimicrobial metabolites

Antimicrobial metabolites are secondary metabolites belonging to heterogenous groups of organic compounds produced by microorganisms that affect the growth or metabolic activities of other microorganisms. Antibiotics are largely produced by bacteria and fungi (Köhl, Kolnaar et al. 2019).

Since a very small fraction of bacterial biodiversity can be cultivated on usual culture media, it can be assumed that the majority of *in situ* produced antibiotics are unknown. Production of antimicrobial metabolites has been reported for bacteria belonging to the genera *Bacillus, Pseudomonas, Serratia, Streptomyces* and many others (Köhl, Kolnaar et al. 2019).

1.9.2. Indirect interactions with plant pathogens

Competition for nutrients and space

Necrotrophic pathogens interact with the host by invading its tissue, leading to damage. However, most pathogen populations spend a significant part of their development living as saprophytes on necrotic plant tissues, crop residues and residues of non-hosts. During this stage, the host is unnecessary for some species, while others may still require the presence of the host in order to complete their life cycle. Due to the saprophytic stage, where the pathogens depend on exogenous nutrients, they become vulnerable to nutrient competition. As a result, highly competitive organisms can be utilized as biological control agents (Köhl, Kolnaar et al. 2019).

Besides competition for carbohydrates and nitrogen sources, restricted iron availability can impact the development of the microorganisms. In 1995, Raaijmakers et al. used *Pseudomonas sp.* to demonstrate that iron competition resulted in reduced pathogen populations (Raaijmakers, Sluis et al. 1995).

Induced resistance and priming

Plants possess a large variety of mechanisms that allow them to protect themselves from pathogens. Their defense mechanisms are triggered by stimuli produced by either the pathogens or beneficial microorganisms (Köhl, Kolnaar et al. 2019).

Pathogen-Associated Molecular Patterns (PAMP) induce defense pathways in the plant to increase its resistance, resulting in Systemic Acquired Resistance (SAR), which is induced by necrotizing pathogens (Conrath, Beckers et al. 2015).

Microbe-Associated Molecular Patterns (MAMP) are stimuli produced by growth-promoting bacteria and fungi and lead to Induced Systemic Resistance (ISR) in the plant (Conrath, Beckers et al. 2015).

Induced defense mechanisms in plants are the production of reactive oxygen species and phenolic compounds, along with modifications of cell walls and cuticles by the induced plant (Köhl, Kolnaar et al. 2019).

1.10. *Fusarium species. Fusarium culmorum*

Fusarium is a genus of filamentous fungi that can be found in plants and soils. The genus contains both saprophytic and pathogenic species (Patil, Sriram et al. 2011). Cultures of *Fusarium* can appear yellow, orange or red depending on the strain, maturity, nutrients, temperature, pH and light exposure. The color of *Fusarium* can be attributed to the accumulation of pigments in the cell wall, such as neurosporaxanthin (either orange or yellow), aurofusarin (yellow) and rubrofusarin (red). The color can be used to identify *Fusarium* infections on plants (Cambaza 2018).

Fusarium culmorum is a ubiquitous soil-borne fungus with a highly competitive saprophytic ability. However, *F. culmorum* is also a severe pathogen that can cause both Fusarium Head Blight (FHB), also known as "head scab" and Fusarium Crown Rot (FCR), also known as Fusarium Seedling Blight (FSB) or Fusarium Root Rot (FRR), two diseases in small grain cereal. Fusarium crown rot causes damping off following infection of the leaf sheaths, stem base and roots of mainly seedlings and early stage plants (Rojas 2019). The most important disease is FHB, which causes reduction in yield and quality of cereals such as barley, oat and wheat (Bertero, Moretti et al. 2018). *Fusarium culmorum* is also known as a post-harvest pathogen, mainly on freshly harvested grain that was improperly stored or dried (Aldred and Magan 2004; Eifler, Martinelli et al. 2011).

In terms of variability, two chemotypes of *F. culmorum* have been described: chemotype I, which produces deoxynivalenol (DON) and its acetylated derivates (3-ADON, 15-ADON), and chemotype II, which produces nivalenol (NIV) and fusarenone-X (FUS). Nivalenol is approximately 10 times more toxic than deoxynivalenol (Minervini, Fornelli et al. 2004).

Table 5 presents the geographical distribution of the *F. culmorum* chemotypes (Tab. 5).

Table 5. The geographical distribution of *Fusarium culmorum* chemotypes (Scherm, Balmas et al. 2013).

Country	Chemotyping method used	Number of isolates analyzed	Main finding	Reference

Europe	Chemical	42	~84% DON producers, ~16% NIV producers	(Gang, Miedaner et al. 1998)
Germany	Chemical	27	~60% NIV producers, ~40% DON producers	(Muthomi, Dehne et al. 2000)
Norway	Chemical	23	Mostly 3-ADON producers, two NIV producers	(Langseth, Ghebremeskel et al. 2001)
France	Genetic and chemical	60	58% NIV producers, 42% DON producers	(Bakan, Pinson et al. 2001; Bakan, Giraud-Delville et al. 2002)
Denmark, Germany, Austria	Chemical	102	1995 sampling: ~90% DON producers, ~10% NIV producers	(HESTBJERG, FELDING et al. 2002)
The Netherlands	Genetic	85	2000–2001 sampling: mostly NIV producers	(Waalwijk, Kastelein et al. 2003)
Worldwide (Australia, Canada, Israel, Hungary, Germany, Denmark, the Netherlands, Morocco)	Genetic and chemical	37	19% NIV producers, 81% 3-ADON producers	(Tóth, Mesterházy et al. 2004)
UK	Genetic	157	DON producers are prevalent, but NIV producers are distributed consistently	(Jennings, Coates et al. 2004)
Europe (Spain, Italy, Poland, Norway, the	Genetic	55	~20% NIV producers, ~80%	(Quarta, Mita et al. 2005)

Netherlands, France, Finland, former Yugoslavia)			3-ADON producers	
Belgium	Genetic	128	In 2007 (95%) and in 2008 (88%) NIV producers are the most diffused	(Audenaert, Van Broeck et al. 2009)
Luxembourg	Genetic and chemical	175	3-ADON and NIV producers are evenly distributed	(Pasquali, Giraud et al. 2010)
Tunisia	Genetic and chemical	100	Mostly 3-ADON producers, 2% NIV producers	(Kammoun, Gargouri et al. 2010)
Poland	Genetic	68	6% NIV producers, 94% 3-ADON producers	(Baturo-Ciesniewska and Suchorzynska 2011)
Turkey	Genetic	21	100% 3-ADON producers	(Yörük and Albayrak 2012)

Fusarium sp. are among the predominant fungi responsible for the contamination of cereal grains with mycotoxins, and as such posing a risk to animal and human health. It is estimated that about 25% of global cereal production may be contaminated with mycotoxins (Cuomo, Güldener et al. 2007). In the case of *Fusarium culmorum*, it can cause grain contamination with type B trichothecenes, zearalenone and fusarins. Sesquiterpene epoxide trichothecenes are considered the most bioactive compounds produced by *F. culmorum*, and they are able to inhibit eukaryotic protein synthesis, induce apoptosis, act as virulence factors and cause toxicosis in humans or animals consuming contaminated food or feed (Scherm, Balmas et al. 2013).

1.11. Fusarium Crown Rot

Fusarium crown rot symptoms can vary depending on the time of infection. In the case in which the fungus attacks at an early stage, for example just after sowing, pre- and post-emergence seedling death

occurs, with brown discoloration being present on the roots, coleoptiles and pseudo stem. These symptoms describe Fusarium Seedling Blight (FSB) (Fig. 9)(Jensen, Knudsen et al. 2000).

Figure 9.Symptoms of FSB; brown discoloration (right) and normal aspect of roots and coleoptiles (Pscheidt 2020).

In the case in which the infection starts later in the season, brown lesions are present on the first two or three internodes of the main stem (Fig. 10). In high humidity conditions, a reddish-pink discoloration can be found on the nodes due to the presence of sporulating mycelium (Fig. 11)(Scherm, Balmas et al. 2013).

Figure 10. Brown lesions on the internodes of the main stem (Scherm, Balmas et al. 2013).

Figure 11. Reddish-pink discoloration on the nodes (Scherm, Balmas et al. 2013).

FFR caused by *F. culmorum* increases in severity in warm areas, where the plant might be subjected to water stress. This is attributed to the sensitivity of the plant towards the fungal pathogen due to draught conditions rather than an increased virulence of the fungus in these areas (Chekali, Gargouri et al. 2011).

1.12. Fusarium Head Blight (FHB)

Fusarium Head Blight (FHB) is one of the most devastating fungal diseases of grain crops such as wheat, barley and maize (Dweba, Figlan et al. 2017).

Symptoms of FHB include partial head blighting, with the bleaching of one or more of the spikelets (Fig. 12), or the bleaching of the whole head (Fig. 13) (Scherm, Balmas et al. 2013).

Figure 12. Symptoms of FHB; partial head blighting (Scherm, Balmas et al. 2013).

Figure 13. Symptoms of FHB; blighting of the entire head (Scherm, Balmas et al. 2013).

1.13. Environmental conditions

Fusarium culmorum is present mostly in northern, central and western Europe. However, the incidence of *Fusarium graminearum* is on the rise in these areas. The switch might be explained by the crop rotation of wheat with feed maize in northern Europe, since while *F. culmorum* has a wide range of host plants and it was also isolated from maize crops and kernels, it was not the main pathogen (Scauflaire, Mahieu et al. 2011). Another explanation could be in the adaptation of *F. graminearum* to colder climates due to genome plasticity or the overall rise in temperatures caused by climate change (Raffaele and Kamoun 2012; West, Holdgate et al. 2012).

Contrary to the well-established geographical distribution, in 2011, Fakhfakh et al. isolated spore cultures from durum wheat fields in Northern Tunisia. Out of all the isolates, *Fusarium culmorum* was the predominant species, representing 36.3% of all sampled isolates (Fakhfakh, Yahyaoui et al. 2011).

1.14. Aim and approaches

This project, done in collaboration with Copenhagen University, aims at developing effective bioaugmentation strategies that would both lower the persistence of the pesticide Dicamba in contaminated soils and provide biological control against fungal pathogens in cereal crops, which could potentially decrease the necessity of certain compounds being applied on the crops or providing

enhanced features to the plants, such as protection against draught or resistance against high pesticide concentrations. If the bacterial species in the soil microbiome could degrade the applied or drifted pesticides, the pesticide impact on sensitive plants would greatly decrease.

The first part of the project was performed in Aalborg University Copenhagen and involves the determination of the best pesticide degraders from the available isolated strains. In order to determine the degrading efficiency of the isolates, a Pesticide Degradation Assay was employed, by inoculating M9 minimal media, with Dicamba as sole carbon source, with the preactivated bacterial isolates, and incubating for a period ranging from 2 weeks to 1 month. The degradation amount is determined through HPLC analysis.

A separate experiment utilizes 4 different microbial consortia, which will be tested through the same Pesticide Degradation Assay.

Organisms will also be plated on a selective medium where only pesticide-degrading bacteria should thrive, and quantification of the degraded amount from solid medium will be performed.

Based on the results obtained from the best degraders among the isolates, some organisms might also exhibit biological control abilities, which will be tested using a sand assay with wheat seedlings and *Fusarium culmorum* as a fungal pathogen. This part of the study has been performed in Copenhagen University.

The main aim of the study is to find organisms that possess dual function: being able to efficiently degrade the pesticide Dicamba, and at the same time exhibit biological control abilities against the fungal pathogen.

2. MATERIALS AND METHODS

2.1. Chemicals and solutions

The pesticides Malathion, Dimethoate and Dicamba were provided in standard form by FMC Corporation. They were stored in a ventilated cupboard at room temperature. Along the project, in the labeling of the different conditions, and the subsequent isolated strains, they are referred respectively as Mal, Dim and Dic.

After the first Dicamba reserve was depleted, Dicamba analytical standard was obtained from Sigma Aldrich.

In order to prepare the Mineral Minimal Media (M9 medium) used, a stock solution of M9 salts was prepared. To make M9 Salts, 800 mL of H_2O were aliquoted, to that 64 g Na_2HPO_4-$7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl and 5.0 g NH_4Cl were added, stirred until dissolved, adjusted to 1 liter with distilled H_2O and then sterilized by autoclaving. The other two solutions made, $MgSO_4$ 1M and $CaCl_2$ 1M, were prepared and autoclaved separately. To prepare M9 media, 700 mL of distilled H_2O were aliquoted in a 1 L bottle, to which 200 mL of M9 salts, 2 mL of $MgSO_4$ 1M and 100 µL of $CaCl_2$ 1M were added. The volume was then adjusted to 1 L with distilled H_2O. To 1 liter of M9 media, 50 mg of Dicamba were measured using an analytical scale and added, in order to obtain a concentration of 50 mg/L pesticide, which acted as carbon source.

A more nutritive medium was devised through modification of the M9 minimal medium, by addition of Thiamine (0.01 g/L), Biotin (0.03 g/L), Cysteine (1.3 g/L), Ascorbic Acid (0.5 g/L) and SL-10 trace element solution. The medium will be further referred to as Enhanced M9 Medium.

A solid medium was devised from the Enhanced M9 Medium recipe as well, by adding 15 g/L of Bacteriological Agar. This medium will be further referred to as Enhanced M9 Agar.

Trace element solution SL-10 contains HCl (25%; 7.7 M) 10.00 ml; FeCl2 x 4 H2O 1.50 g; ZnCl2 70.00 mg; MnCl2 x 4 H2O 100.00 mg; H3BO3 6.00 mg; CoCl2 x 6 H2O 190.00 mg; CuCl2 x 2 H2O 2.00 mg; NiCl2 x 6 H2O 24.00 mg; Na2MoO4 x 2 H2O 36.00 mg; Distilled water 990.00 ml.

The LB medium used contained tryptone, 10 g/L; yeast extract, 5 g/L; and NaCl, 10 g/L. LB-agar was made adding 15 g/L agar to LB medium.

Yeast Extract M9 was utilized as intermediary medium in the improved Pesticide Degradation Assay. It is represented by an Enhanced M9 Medium with 0.3 g/L of Yeast Extract and 50 mg/L of Dicamba.

M12 medium was used during the medium optimization step of the experiments. M12 Medium contains K_2HPO_4 2.59 g/L, KH_2PO_4 1.3 g/L, $(NH_4)_2SO_4$ 2 g/L, $MgSO_4$*$7H_2O$ 0.2 g/L, $CaCl_2$*$2H_2O$ 0.02 g/L and $FeSO_4$*$7H_2O$ 0.005 g/L.

RA medium was utilized for *Fusarium culmorum* sporulation, in order to make spore suspensions for the Dual Plate assay. RA medium contains succinic acid 50 g/L; $NaNO_3$ 12.1 g/L; Glucose 1g/L; Vogels salts 50x 20 ml/L; completed with MiliQ water until the 1-liter mark. The solution is then autoclaved.

Vogels salts 50x: Na_3-citrate*$2H_2O$ 125 g/L; KH_2PO_4 250 g/L; $MgSO_4$*$7H_2O$ 10 g/L; $CaCl_2$*$2H_2O$ 5 g/L; Trace solution 200X 5 mL; MiliQ water added to the 1-liter mark. The solution is filter sterilized.

Trace solution 200X: Citric acid*H_2O 5 g; $ZnSO_4$*$7H_2O$ 5 g; $Fe(NH_4)_2(SO_4)_2$*$6H_2O$ 1 g; $CuSO_4$*$5H_2O$ 0.25 g; $MgSO_4$*H_2O 0.05 g; H_3BO_3 0.05 g; Na_2MoO_4*$2H_2O$ 0.05 g; MiliQ water added to the 100 mL mark. The solution is filter sterilized.

Potato Dextrose Agar (PDA) 1/3 was utilized in the Dual Plate assays, as it is a suitable substrate for both the fungus and the bacteria. Only 1/3 was used in order to make the medium less nutritive, in an attempt to balance the growth of the fungus and bacteria. PDA 1/3 medium: Potato Dextrose 8 g/L; Agar 15 g/L; For PDA 1/3 with Dicamba, the preparation of the media is the same, with the exception that 50 mg/L of Dicamba are added.

2.2. Organisms

The samples that resulted in the bacterial isolates originate from previous work and were previously taken from three different locations, and are referred to as FS (FMC Soil), coming from five different locations in the surrounding area of the Cheminova pesticide formulating facility in Rønland, HS (Hungary Soil), coming from a pesticide contaminated agricultural field in Hungary and SLU (Activated Sludge), provided by FMC Corporation and obtained from the wastewater treatment plant of the Cheminova pesticide formulating facility. The samples then underwent 6 enrichment steps with the same pesticide concentration in M9 medium, for a total of 11 weeks, with 3 initial 1-week steps, a 4^{th} 2 weeks step and two last 3 weeks steps. The concentrations used were 750 mg/L for Dicamba, 150 mg/L for Dimethoate and 300 mg/L for Malathion. At the end of the enrichment, the organisms present were isolated and frozen at -80 °C.

Four pesticide-contaminated soil samples were chosen for enrichment of Mixed Microbial Consortia (MMC), three of them from soil taken from the Cheminova pesticide formulating facility in Rønland (FMC MP, FS and FMC WEST) and one taken from a field contaminated with pesticides from Hungary by an Aalborg University professor (HS). Each of the consortia that resulted kept the name of the place the soil samples came from. As such, the four MMCs will be further referred to as FMC MP, FS, FMC WEST and HS.

From the AAU organism library, *Bacillus subtilis, Pseudomonas putida* and *Micrococcus* luteus were utilized in further experiments, three bacterial species that have been known to be able to degrade

pesticides (Sims, Sommers et al. 1986; Gilani, Rafique et al. 2016; Gangola, Sharma et al. 2018; Jiang, Zhang et al. 2019)

The MMC that was utilized in further experiments in this paper is the fifth step of enrichment from previous work, as it had the highest biodiversity of all the steps, proven by the metagenomics studies performed.

The nomenclature of the isolates is as follows: the origin of the sample (HS, FS, SLU), the pesticide they were enriched on (DIC, DIM, MAL) and an assigned number (ex. SLUMAL222).

From the KU laboratories, *Pseudomonas putida KT2440* provided by Mette H. Nicolaisen, University of Copenhagen, PLEN Department and *Fusarium culmorum* strain 5 were used (Jensen, Knudsen et al. 2000). *Pseudomonas putida KT2440* was used for the first sand assay, and *Fusarium culmorum* strain 5 was used for all the sand assays as the fungal plant pathogen.

2.3. Enrichment of soil bacteria

Four pesticide contaminated soil samples named FMC MP, HS, FMC Soil and FMC WEST were chosen for development of four mixed microbial consortia.

From each of the four soil samples, 5 grams of soil were weighed using an analytical scale, then taken and added into 20 mL of Enhanced M9 Medium with 50 mg/L Dicamba in cotton-capped 100 mL Erlenmeyer flasks. The vessels were incubated in a shaking incubator at 150 rpm, 30 °C.

Every 7 days, 2 mL were taken from the flask and used as inoculum in another Erlenmeyer flask with 18 mL of Enhanced M9 Medium with pesticide. This step was repeated 4 times in order to wash out the soil, so that the carbon source is limited to the pesticide. Every 7 days, the previous passage was frozen.

From each vessel, samples of 100 µL were extracted every 1-2 days, for measurements using the Microbial Cell Viability Assay BacTiter-Glo™. The cultures were assessed at the end of Passage 4 based on how high their metabolic activity values were.

2.4. Assessing the cell viability of the organisms: BacTiter-Glo™

BacTiter-Glo™ Microbial Cell Viability Assay (Promega) is a homogeneous method for determining the number of viable bacterial cells in culture based on quantification of the ATP present. ATP is an indicator of metabolically active cells. The assay procedure involves adding a single reagent (BacTiter-GloTM Reagent) directly to bacterial cells in medium and measuring luminescence. The formulation of the reagent supports bacterial cell lysis and generation of a luminescent signal. The luminescent signal is proportional to the amount of ATP present, which is directly proportional to the number of active cells in the culture (Promega 2019).

The BacTiter-Glo™ Reagent uses a thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) and a method of extracting ATP from bacteria. The assay generates a "glow-type" luminescent signal, produced by the luciferase reaction which has a signal half-life generally over 30 minutes depending on the bacterium and medium. 100 µl of sample were added to an Eppendorf tube along with 100 µl of BacTiter-Glo™ reagent and analyzed. BacTiter-Glo™ reagent was prepared mixing both reagents equilibrated at room temperature. One of the reagents consisted in a buffer and the other consisted in a powder-like substrate mix. The 10 mL of the buffer were added to the bottle of the substrate, mixed and equilibrated for at least 15 minutes at room temperature. The luminescence was recorded with a GloMax® 20/20 Luminometer (Promega 2019).

2.5. HPLC-UV

The HPLC system was composed of an Altex model l00A solvent delivery pump (Berkeley, CA) and a Hitachi 100-40 UV-detector (Danbury, CT) fitted with an Altex spectrophotometer flow cell set at 229 nm (range, 0.2; time constant, 0.3). The retention data and peak areas were recorded and analyzed by a Hewlett-Packard H3396 A integrator (Avondale, PA) which had the following settings: attenuation, 32; peak width, 0.2; threshold, 1.0; and area rejection 100. The HPLC column used was a commercially available Phenomenex ODS (4.6 mm x 150 mm) with Spherisorb packing consisting of a 5.0 pm pore size (Torrance, CA)(Fogarty, Traina et al. 2006).

The mobile phase consisted of 40% acetonitrile-60% phosphate buffer (6.0 g K_2HPO_4 and 3.0 mL conc. H_3PO_4 pH 3.0) (Fogarty, Traina et al. 2006).

Working standards for HPLC analysis of Dicamba were prepared in 0.05 M NaOH by making two stock solutions: a 1 liter 0.05 NaOH solution, and 1 liter of a 0.05 M NaOH solution with 1 gram of Dicamba and combining them in different proportions. The Dicamba powder was weighed using an analytical scale and then added into the 1L 0.05 M NaOH solution. Since Dicamba is soluble in water, agitation of the bottle was enough to ensure proper mixing. The proportions for the standards are as follows:

Table 6. Standard preparation proportions for the pesticide Dicamba.

DICAMBA STANDARDS CONCENTRATION	0.05 M NaOH WITH 1 g/L DICAMBA	0.05 M NaOH
5 mg/L	500 µL	99.5 mL
25 mg/L	2.5 mL	97.5 mL
50 mg/L	5 mL	95 mL
75 mg/L	7.5 mL	92.5 mL

100 mg/L	10 mL	90 mL

The sample preparation consisted solely on filtering the 1 mL samples through a 0.45 μm Acrodisc LC25 filter.

All standards and samples were filtered through a 0.45 μm Acrodisc LC25 filter (Gelman Sciences, Ann Arbor, MI) before injection (10 μl) into HPLC. Analyses were carried out under isocratic conditions at a flow rate of 1 mL min^{-1} and chart speed of 0.5 cm min^{-1} (Fogarty, Traina et al. 2006).

2.6. Pesticide Degradation Assay

Prior to starting the experiment, the isolates were defrosted from -80℃ and preactivated in LB medium. After 24 hours, three samples of 2 mL were taken from each of the preactivation vessels and added as inoculum to three other vessels which contained 18 mL of LB medium, leading to a working volume of 20 mL. After using the preactivation vessels as inoculum, the rest was frozen at -20℃ in order to have the cells readily available.

After 24 hours, for each organism the three vessels were analyzed with a spectrophotometer at 600 nm to determine optical density, and as a result, the vials with the highest bacterial titer were chosen. Depending on the results, one, two or all three vessels could be used as inoculum for the Degradation Assay.

The samples of the Pesticide Degradation Assay were cultivated and incubated in 100 mL serological vials with cotton caps, in a total working volume of 20 mL consisting of 18 mL M9 medium with 50 mg/L of Dicamba, and 2 mL bacterial inoculum. The negative controls consisted of 18 mL M9 medium with 50 mg/L of Dicamba and 2 mL LB medium. They were incubated in a shaking incubator at 150 rpm and 30℃ for two weeks. During day 1, day 7 and day 14, HPLC samples were taken in order to determine the degradation amount. The experiment was performed in duplicates.

The Pesticide Degradation Assay is employed in order to determine the best pesticide degraders.

After the preliminary testing of the isolates, several steps were taken in order to increase accuracy and efficiency of the method. Firstly, the working volume has been changed from 20 mL to 50 mL in order to minimize error. At the same time, evaporation as a variable was removed, as the cotton caps were replaced with parafilm. During a 2-week test, no evaporation occurred through incubation at 30°C while the vessels were parafilmed, since parafilm is permeable to gases, but highly insensitive to moisture loss.

The M9 medium was replaced with Enhanced M9 medium, in order to improve the growth conditions of the organisms, as this medium contains important vitamins and trace elements.

Since it is a possibility that organisms might not need to utilize the pesticide since 2 mL of LB inoculum are present in each vessel, a strategy was devised to limit the presence of nutrients and at the same time provide a better shift from a highly nutritive medium to a nutritive medium. Firstly, after the initial growth of the organisms in LB medium, instead of being transferred directly to the working medium, 2 mL were taken from the LB vessels to be added as inoculum to 18 mL of Yeast Extract M9, which acted as in intermediary medium. The organisms were grown for 2 days on the intermediary medium, then they were centrifuged at 4000 rpm for 7 minutes, and washed 3 times, in order to ensure minimal presence of nutrients. Then, 5 mL of washed inoculum were added to 45 mL of Enhanced M9 medium with 50 mg/L of Dicamba for the long-term experiments.

2.7. The effect of different pesticide concentrations on bacterial degradation

In order to determine the best pesticide concentration for degradation optimization, 3 different Dicamba concentrations were chosen: 50 mg/L, 150 mg/L and 250 mg/L. The experiment was performed in 50 mL Falcon Tubes with cotton caps, at a working volume of 20 mL, with an inoculum of 2 mL preactivated in LB, in duplicates. The negative controls were represented by M9 medium with the same pesticide concentrations, but without the bacterial inoculum, instead two milliliters of LB medium were added to the M9 medium controls. They were incubated in a shaking incubator at 150 rpm and 30 °C for three weeks. During day 1, day 7, day 14 and day 21, HPLC samples were taken in order to determine the degradation amount. The isolate chosen for this assay was HSDIC24, since it showed degradation of Dicamba during the first round of the Pesticide Degradation Assay.

2.8. Effect of different inoculum sizes in quantification of Dicamba degradation

In order to improve the parameters of the degradation experiments, different inoculum sizes were also tested. With an increased inoculum size in the same volume with the same available carbon source, the amount of the pesticide that is degraded should increase. The experiment was performed in 250 mL serological vials, in a working volume of 50 mL.

The organism selected for this experiment was FSMAL211, since it showed degradation abilities (tested in Round 4 of the Pesticide Degradation Assay). Three conditions were tested, 10% inoculum size (5 mL in 50 mL total volume), 20% (10 mL inoculum in 50 mL total volume) and 30% (15 mL inoculum in 50 mL total volume). A control condition was also present, which contained 50 mL of Enhanced M9 Medium with 50 mg/L Dicamba, but without a bacterial inoculum.

The organism FSMAL211 was taken from -80 °C and pre-activated in LB medium for 24 hours. After the 24-hour mark, it was centrifuged at 4000 rpm for 7 minutes, the supernatant was thrown, and the pellet was resuspended in Enhanced M9 Medium with 50 mg/L of Dicamba. This step was performed 3 times in order to ensure that the nutrient leftovers from LB are minimal. After the washing steps, the vessels were added the required inoculum volume for the 3 conditions, and they were parafilmed to avoid evaporation. The samples were incubated in a shaking incubator at 30°C, 150 rpm for 2 weeks, and HPLC samples were taken in the first day (day 1) and the final day (day 14) of the experiment. The experiment was performed in duplicates.

2.9. Growth of pesticide degrading organisms on solid medium; Recovery of the pesticide Dicamba from solid medium

In order to better observe the cultures and to develop a quick assay to determine which of the organisms are capable of degrading Dicamba, a selective solid medium was devised that would only allow the pesticide-degraders to grow, the Enhanced M9 Agar medium. Organisms added to the Petri dishes came from liquid cultures, as such they needed to be washed of any leftover nutrients.

The liquid cultures were centrifuged at 4000 rpm for 7 minutes, the supernatant was discarded and the pellet was resuspended in the same amount of M12 medium, since it has a low salt concentration and as such accumulation of salts in the pellet that would lead to increased osmotic pressure would not pose a risk. This washing process was repeated 4 times in order to ensure proper removal of leftover liquid nutritive medium.

After the washing steps, 200 µL of the resulted liquid is added to an Enhanced M9 Agar plate and then spread. After 1-week incubation, if colonies are present on the plate, a colony is picked and re-streaked on another Enhanced M9 Agar plate to ensure increased accuracy of the results.

Since there is a strong possibility that some organisms may be able to utilize other constituents of the medium other than Dicamba (for example the agar itself), a control medium was devised, a medium that has the same composition as the Enhanced M9 Agar, but without the addition of 50 mg/L of Dicamba. The plates were inoculated in the same manner as the Enhanced M9 Agar plates explained above and treated in the same manner, being incubated for the same amount of time (1 week), at the same temperature (30 °C), on the same shelf of the incubator.

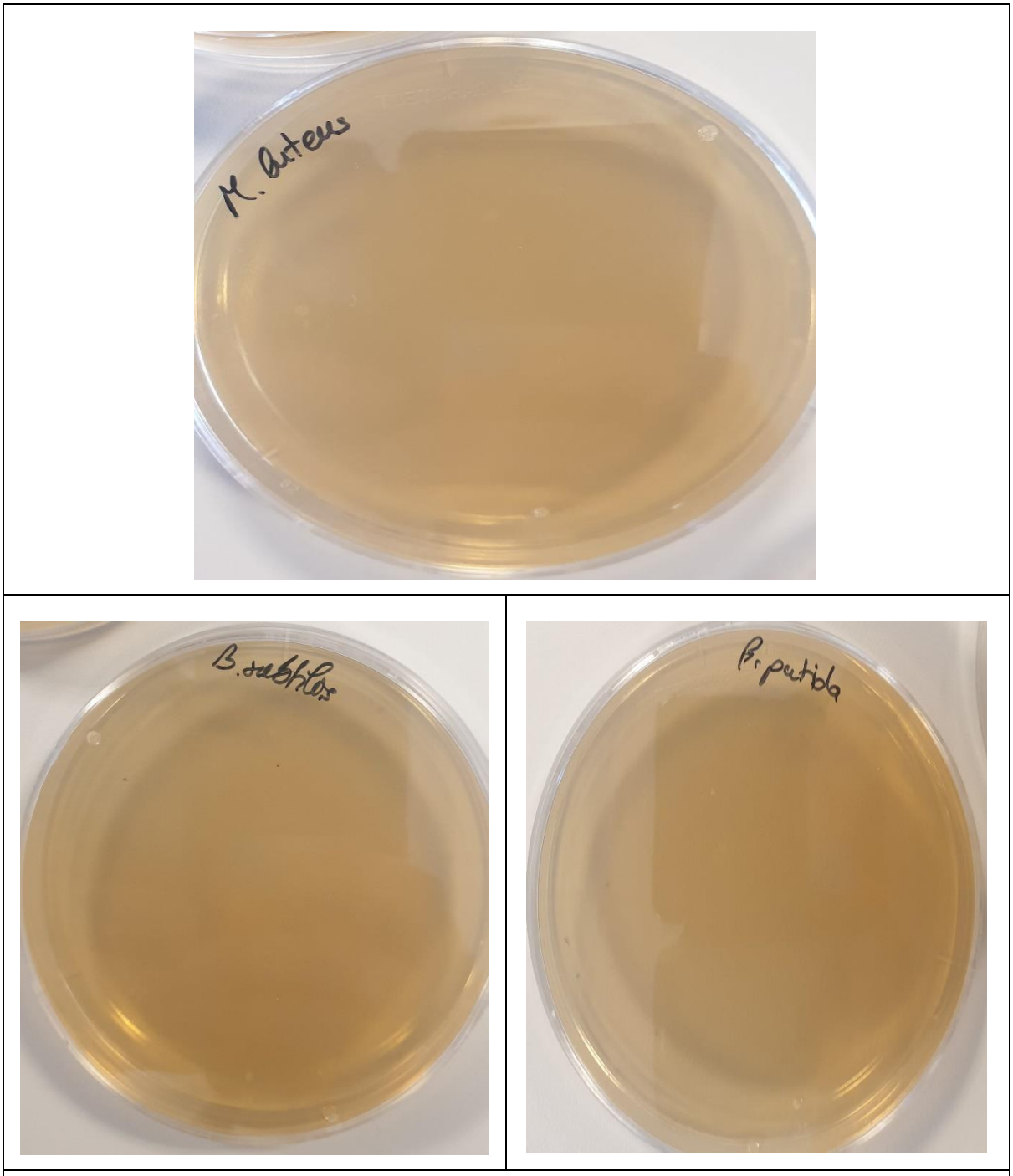

Figure 14. Control plates of Enhanced M9 Agar without the 50 mg/L Dicamba, inoculated with *Micrococcus luteus*, *Bacillus subtilis* and *Pseudomonas putida*, after one week of incubation.

On the plates without Dicamba, no growth has been observed.

In order to determine the amount of Dicamba utilized by the organisms, an assay was developed to quantify the Dicamba concentration present in the Enhanced M9 Agar plates. The agar plates are frozen at -20 °C overnight, and then thawed. After the thawing step, the gel is added to 50 mL Falcon Tubes and centrifuged at 5000 rpm for 15 minutes. Through centrifugation, a significant amount of supernatant is present, which can then be sampled and analyzed through HPLC.

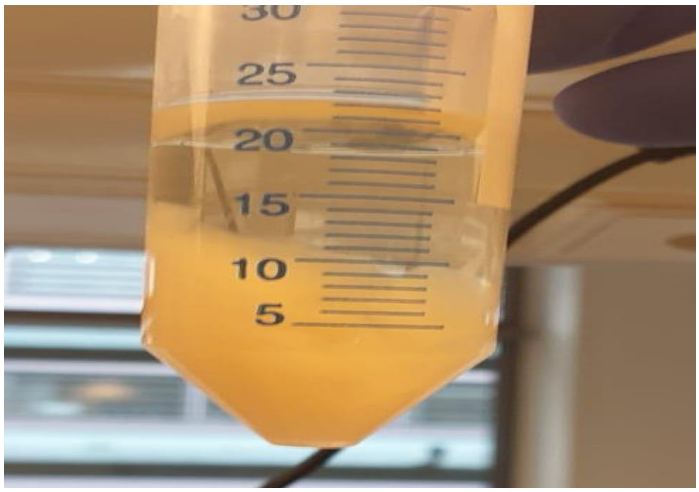

Figure 15. An Enhanced M9 Agar plate after freezing, thawing and centrifugation at 5000 rpm for 15 minutes.

The recovery rate of Dicamba in the supernatant is tested using a non-inoculated Enhanced M9 Agar plate, taken through the same steps mentioned above, and then the quantity of Dicamba present in the supernatant is determined through HPLC analysis.

2.10. Sand assay to evaluate the biocontrol efficacy of seed coating with bacterial isolates against *Fusarium* seedling blight

The wheat cultivar Diskett was used in all conducted sand assays. The protocols used for setting up the Fusarium Seedling Blight assay and for evaluation of disease severity are based on methods described by Jensen et al (Jensen, Knudsen et al. 2000). In this assay, wheat seeds are sown in plastic pots that are filled with a sand and water mixture. The seeds are inoculated with either a biological control agent, a fungal pathogen or both and incubated in a growth chamber for 2 weeks. Two experiments were performed. The first experiment used *Pseudomonas putida* strain KT2440 as biological control agent at two different concentrations, while the second experiment utilized *Bacillus subtilis, Micrococcus luteus* and a different strain of *Pseudomonas putida*, from the AAU laboratories as biological control agents. Two different seed lots were used in the two experiments, one with a low germination rate for the first experiment and one with a high germination rate for the second experiment.

The first step, before inoculation of the seeds with either the fungal pathogen, the biological control agent or both, is the surface sterilization of the seeds. The seeds are dipped in 70% ethanol for 20 seconds, and then sieved to separate them from the liquid and moved into a vessel containing 2.5% Sodium Hypochlorite (NaOCl) for 10 minutes. After the 10 minutes mark, the seeds are washed 3 times

in sterile water through the same sieving technique used to separate them from the ethanol, and then dried overnight inside a Laminar Flow Bench set at ½ speed.

Inoculation of seed with *F. culmorum* (strain 5). The pathogen isolate of *F. culmorum* was isolated from a barley seed lot (Jensen, Knudsen et al. 2000). The pathogen is grown on Potato Dextrose Agar (PDA) for 14-21 days. The spores are harvested from plates flooded with water followed by filtration through 2 layers of cheese cloth or a nylon mesh (100 µm). The suspension is adjusted to $1{,}5 \times 10^6 \pm 0{,}5 \times 10^6$ spores/ml. The seeds are inoculated by soaking in the spore suspension (1: 2 w/v) for 30 min on a shaker at 130 rpm and then dried on filter paper for 24 h in a sterile laminar flow chamber. Inoculated seeds can be stored at 4˚C until use. Healthy control seed must be shaken with sterile water in a similar way as described above.

Inoculation of seed with Biocontrol Agents. The bacterial species used were *Pseudomonas putida* strain *KT2440,* provided by Mette H. Nicolaisen, University of Copenhagen, PLEN Department, and *Pseudomonas putida, Bacillus subtilis, Micrococcus luteus* obtained from the Aalborg University Copenhagen laboratories. The bacterial isolates were grown in liquid LB medium, and then the Optical Density of the culture at 600 nm was adjusted to 1 or 1 and 0.1 in the case of *Pseudomonas putida* strain KT2440. The seeds are inoculated by soaking in the bacterial suspension (1:2 w/v) for 10 min on a shaker at 130 rpm.

Seeds infected with *Fusarium culmorum* and treated with various antagonists are sown in sand following the procedure described below.

- Sand and tap water are mixed (3:1 v/v) and strips of four plastic pots are filled with the moist sand mixture. A strip of 4 serial pots with a total of 12 seeds represents a replication.
- Holes are made using a wooden template. Three seeds per hole are sown and covered with sand. Each replicate consists of 12 seeds. Four replicates (4 x 12 seeds) are sown per treatment.
- Pots are placed in saucers and then in trays covered with plastic bags to maintain high humidity, while incubated at 22°C.
- After 8 days, the seedlings are watered in each replicate with 50 ml of water applied to the saucer.
- The roots are washed in water and the severity of seedling blight symptoms is assessed on roots and coleoptiles 14-18 days after sowing following the scale below:

  0 = healthy plant
  1 = slightly brown roots and/or coleoptiles
  2 = moderately brown roots and coleoptiles

3 = severe browning of roots and coleoptiles,

4 = dead plants.

A non-germinated kernel that in infected by Fusarium (red and very soft) is also given the score 4.
A non-germinated kernel without any sign of infection is registered as non-germinated and is not included in the calculation of Disease Index (DI).

Examples of disease score

Table 7. Examples of disease score.

Treatment	Disease score (number of plants per score)					Number of scored plants (including seed killed by Fusarium)	Non-germinated healthy seed
	0	**1**	**2**	**3**	**4**		
Fc	0	3	3	3	2 + 1*	12	0
Fc + BCA1	7	1	1	2	0	11	1
Fc + BCA2	5	3	2	2	0	12	0

The asterisk (*) represents non-germinated kernels infected by Fusarium. ex: out of the three plants with the score 4, two are dead plants and one is a non-germinated Fusarium infected kernel.

The disease index (DI) is calculated by multiplying the number of plants with the respective scores and adding them. The total score is divided by the number of scored plants.

2.11. Sand assay to evaluate the effect of different concentrations of Dicamba on wheat seedlings

The effect of Dicamba on the growth of wheat plants was assessed using the same sand assay described in Section 2.10. with some modifications. The seeds were not inoculated with biocontrol agents or fungal pathogen, while the pesticide Dicamba was added to the sand.

Wheat seeds are sown in sand following the procedure described below.

- Sand and tap water are mixed (3:1 v/v) and strips of four plastic pots are filled with the moist sand mixture. A strip of 4 serial pots with a total of 12 seeds represents a replication.
- Holes are made using a wooden template. Three surface-sterilized seeds per hole are sown and covered with sand. Each replicate consists of 12 seeds. Three replicates (3 x 12 seeds) are sown per treatment.
- Different pesticide concentrations are added to the setup. As such, five conditions are tested: Negative Control, 6.25 mg/L, 12.5 mg/L, 25 mg/L and 50 mg/L. The concentrations were extrapolated and calculated for the surface area of the plant pots, starting from the field application rate of 2 pounds/ acre of Dicamba (Cork and Krueger 1991). The 12.5 mg/L concentration simulates field conditions. It should be mentioned that most of the Dicamba that is applied to field drifts due to wind and as a result, the true concentration on the crops is much smaller than the one theoretically applied.

  In order to obtain these concentrations, a stock solution of 400 mg/L Dicamba was diluted according to the table below. Ten millilitres of each of the solutions were added to the sand pots using a syringe, leading to the presence of 62.5 µg, 125 µg, 250 µg and 500 µg Dicamba in the plant pots.

Table 8. Dicamba concentrations added to the plant setups.

Dicamba 400 mg/L solution (mL)	MiliQ Water (mL)	Final Volume (mL)	Concentration (mg/L)
1.87 mL	118.13 mL	120 mL	6.25 mg/L
3.75 mL	116.25 mL	120 mL	12.5 mg/L
7.5 mL	112.5 mL	120 mL	25 mg/L
15 mL	105 mL	120 mL	50 mg/L

- Pots are placed in saucers and then in trays covered with plastic bags to maintain high humidity, while incubated at 22°C.
- After 8 days, the seedlings are watered in each replicate with 50 ml of water applied to the saucer.
- The roots are washed in water and the effect of Dicamba is assessed on roots and coleoptiles 14-18 days after sowing.

2.12. Dual culture plates

In order to test the potential of different bacteria as biocontrol agents against *F. culmorum*, a dual culture assay was made in which the biocontrol agents and the pathogenic fungi were plated on the same plate. In this way, it is tested whether they can directly inhibit the growth of the pathogenic fungi. This experiment was inspired by a study by Comby et al (Comby, Gacoin et al. 2017).

At the same time, the ability of the bacteria and the fungi to grow on medium that contains the pesticide Dicamba was assessed.

Two lines of the bacteria were streaked out 1.5 cm from the edge of a Petri Dish (94x16mm), containing PDA 1/3 or PDA 1/3 and 50 mg/L of Dicamba, in parallel to each other and a mycelial plug (5 mm in diameter) of 14 days old fungus was placed in the center of the plate. The plates were placed in transparent bags incubated at room temperature near a window, with no direct sunlight. Photos were taken of the plates at day 4, 5, 6, and 7 after plating and the surface area of the fungi was measured using the software ImageJ. The control was represented by the fungal pathogen alone. The *Fusarium culmorum* strain was cultivated on full PDA whereas the bacteria were grown on LB agar.

2.13. Statistical work

Microsoft Excel was used to analyze the acquired data. The following models were used: t-test and ANOVA Single Factor. The data from Section 3.5. and 3.6 was analyzed using ANOVA to determine if there was a statistically significant difference between the disease index of the conditions or the germination rates of the conditions, and then the Bonferroni approach was used to determine which of the conditions were significantly different. The same approach was used to determine the variance in shoot length in section 3.6.

For sections 3.1. and 3.2., only standard deviation was determined and then propagated.

A significance level of 0.05 was used in all models and statistical analysis.

3. RESULTS AND DISCUSSION

3.1. Pesticide Degradation Assay

3.1.1. Dicamba HPLC method

The HPLC method for analysis of Dicamba samples was extracted from (Fogarty, Traina et al. 2006) and modified. The modifications stand in the preparation of the working standards. While in the original method the working standards were diluted in 25 mL of 0.5 M NaOH containing 4 mL glacial acetic acid, in the present method, the standards are simply prepared in 0.05 M NaOH, just like the stock solutions.

The plotted graph of the standards shows a reliable regression line (Fig. 16).

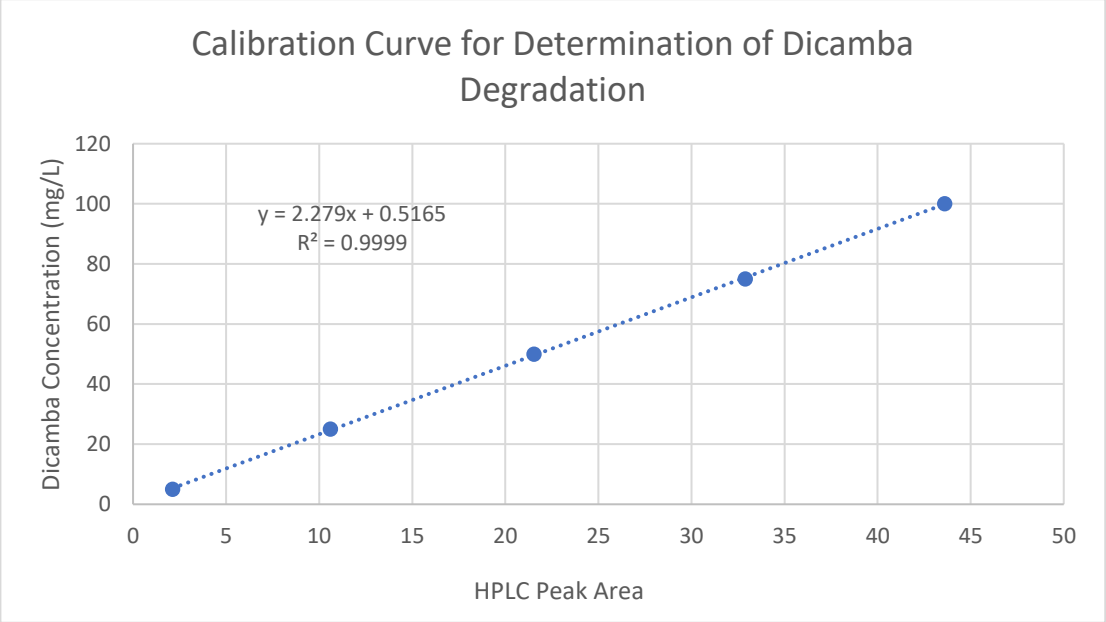

Figure 16. Regression line of dicamba standards in 0.05 M NaOH, with its regression line equation and R-square.

Upon calculation of the concentrations using the HPLC peak area and the y equation (concentration= 2.279*HPLC Peak Area + 0.5165), it can be observed that the calculated concentrations are very close to the standards concentrations (Tab. 9), showing a high fidelity of the method.

Table 9. Calculated concentrations of the Dicamba HPLC Standards.		
Standards	HPLC Peak Area	Calculated Concentrations (mg/L)
Standard 1 (5 mg/L)	2.12	5.35
Standard 2 (25 mg/L)	10.6	24.67
Standard 3 (50 mg/L)	21.54	49.61
Standard 4 (75 mg/L)	32.89	75.47
Standard 5 (100 mg/L)	43.61	99.9

3.1.2. 1st round of organisms

The isolates were tested in rounds of 10 organisms at a time, in order to maximize efficiency and preserve accuracy of the work. In the first round, only 5 organisms were tested (Fig. 17).

In order to determine the degradation amounts, a formula was devised. Due to evaporation of the media during the 2 weeks of incubation, the pesticide Dicamba was up concentrated, so the evaporated amount needed to be considered.

Ci*Vi=Cf*(Vf+ Nr. of samples taken), where

Ci is the initial concentration, Vi is the initial volume, Cf is the final concentration and Vf is the final volume.

The initial concentration is the concentration of Dicamba that accounts for evaporation, and it can be determined by writing the formula as:

Ci= Cf* (Vf+ Nr. of samples taken)/Vi

In this case, Cf is the concentration determined through the HPLC peak area, Vf is the final volume of the flasks at the end of the experiment, after 2 weeks, and Vi is the initial volume of the flasks at the start of the experiment, 19 ml (due to a 1 mL sample for HPLC analysis being taken at the beginning, it is considered that the initial volume is 19 mL instead of 20 mL.

A 3rd control sample was incubated along the other samples, and after the first week its volume was measured. Because of the inability of measuring the actual sample volumes while the experiment was still ongoing, due to possible contamination and increased disturbance, the 1-weeks sample volumes were considered to be the same as the 3rd control sample volume. For the final volumes, there was no need for this procedure, as disturbance no longer represented an issue, and the content of the vessels was extracted and measured using a serological pipette.

In order to determine the degradation amount, certain calculations were performed. Since Dicamba is degraded in the control samples as well due to abiotic factors, that needed to be considered. As a result, in order to determine the microbial degradation amount, the final sample concentration was subtracted from the initial sample concentration, and then the concentration of the control sample was subtracted as well. As a mathematical formula, it is represented as:

(CONC FINAL – CONC I)- CTRL, where:

CONC FINAL is the average final concentration of the duplicates for each sample, CONC I is the average initial concentration of the duplicates for each sample, and CTRL is the (CONC FINAL- CONC I) average value of the control samples. The values were then converted in percentages.

The error bars represent the Standard Deviation of the duplicates that was then propagated for the calculations mentioned above. Through propagation of the error, due to the presence of subtractions and the low overall value of the degradations, the errors shown in the graphical representations are high.

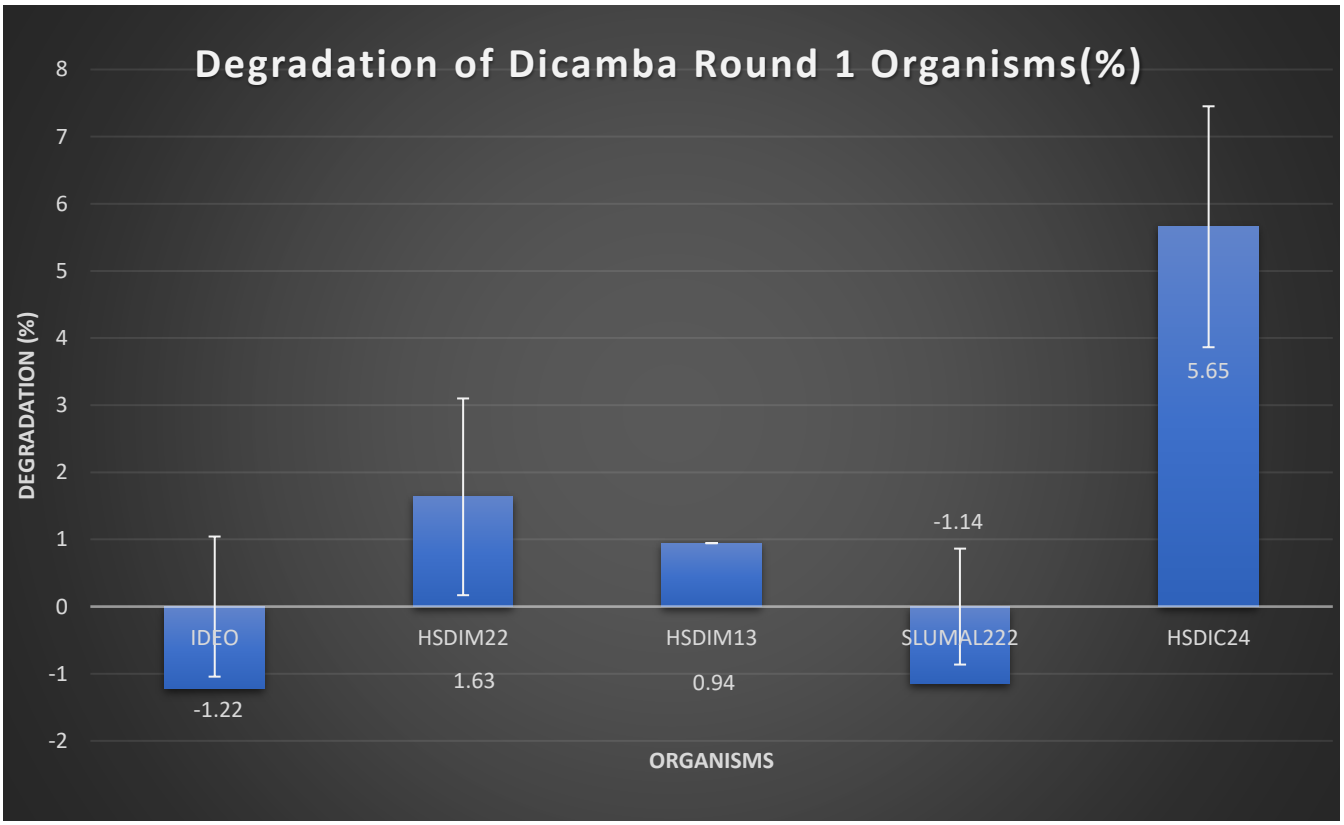

Figure 17. Degradation of Dicamba for the first round of organisms, in percentages. The nomenclature explained above applies, with the exception of IDEO- *Ideonella sakaiensis.* Error bars represent propagated standard deviations.

During the first round, only one of the organisms seemed to be able to degrade the herbicide Dicamba with a degradation of 5.65% compared to the control sample, the organism HSDIC24. According to the nomenclature, this organism was enriched on medium containing Dicamba, which would suggest it should have higher Dicamba degradation abilities than organisms enriched on different pesticides (Dimethoate, Malathion).

3.1.3. 2nd round of organisms

In the second round of organisms, 10 organisms were used, which remains the standard for the duration of the experiments (Fig. 18). As it can be observed, high negative values were registered for some of the organisms. This is due to the high error of the method at this point, since it must account for evaporation, and due to possible contamination of the controls.

The error decreases in further experiments due to optimization of the method. Since the highest degradation value is 1.57% in the case of *Pseudomonas putida*, it is considered that no good Dicamba degraders are present in this round.

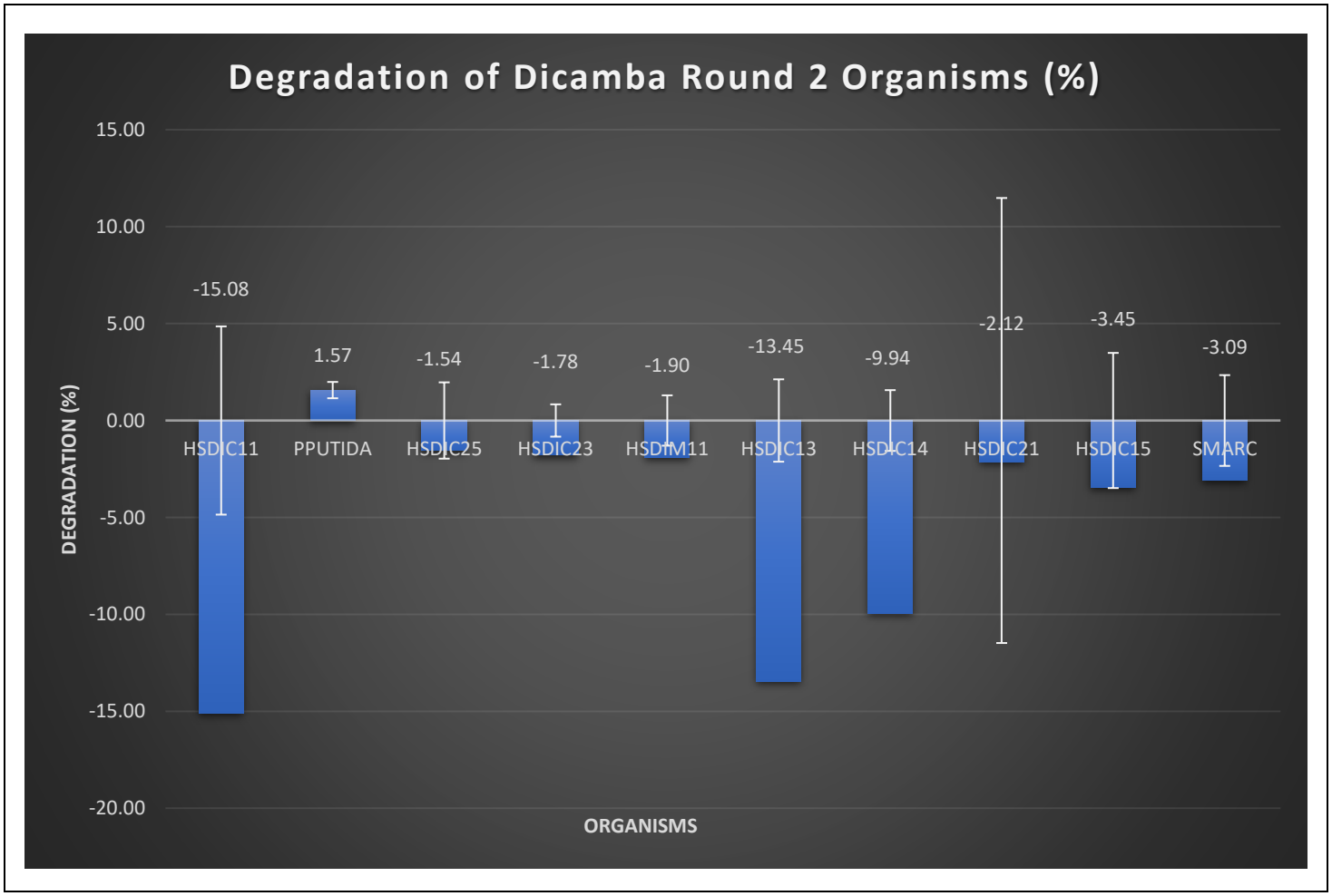

Figure 18. Degradation of Dicamba for the second round of organisms, in percentages.
The organisms keep the same nomenclature explained above, except for PPUTIDA-*Pseudomonas putida* and SMARC-*Serratia marcescens*. Error bars represent propagated standard deviations.

3.1.4. 3rd round of organisms

In the third round of organisms, high negative values were also registered for some of the organisms, while the positive degradation percentages are very low. The highest degradation registered is 1.76% in the case of SLUDIC12. In this experiment, several *Klebsiella sp* were tested (HOLGAK, POLGAK, OOLGAK, GOLGAK), since *Klebsiella sp* have been found to have both pesticide degradation abilities (Kwon, Kim et al. 2002) and biological control function, mainly against nematodes (Liu, Chen et al. 2018) but they do not seem to be able to degrade Dicamba.

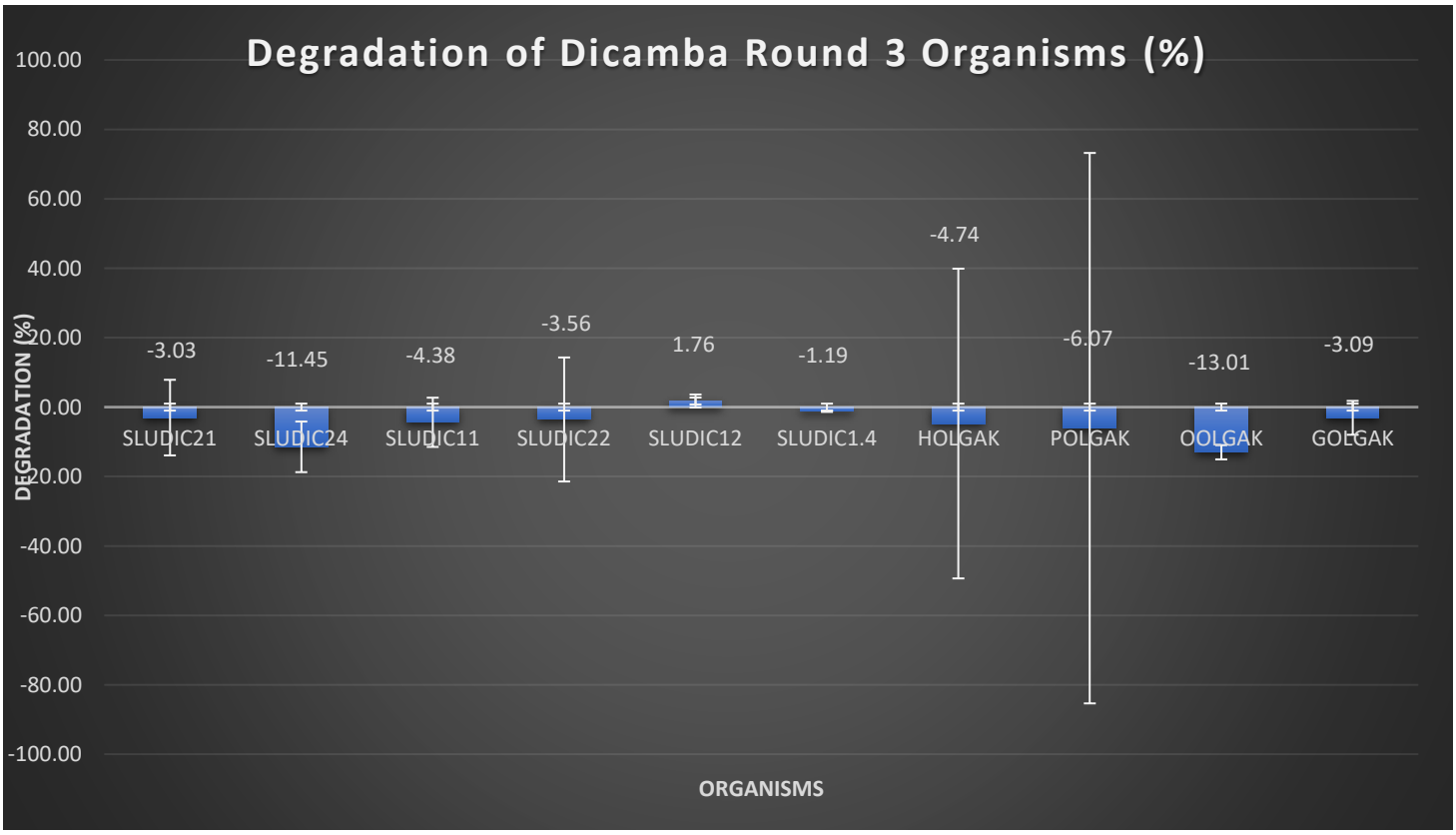

Figure 19. Degradation of Dicamba for the third round of organisms, in percentages. The organisms keep the same nomenclature explained above, except for HOLGAK, POLGAK, OOLGAK and GOLGAK, which are *Klebsiella* species. Error bars represent propagated standard deviations.

3.1.5. 4th round of organisms

In the fourth round of organisms, the negative values have been lowered greatly, the highest negative value in this round being -0.88%, well within the margin of error of the method. In this round, 2 organisms have registered degradation percentages higher than 2%, FSMAL132 with 2.17% and FSMAL211, with 3.06% degradation. Both organisms will be considered for further experiments of increased duration.

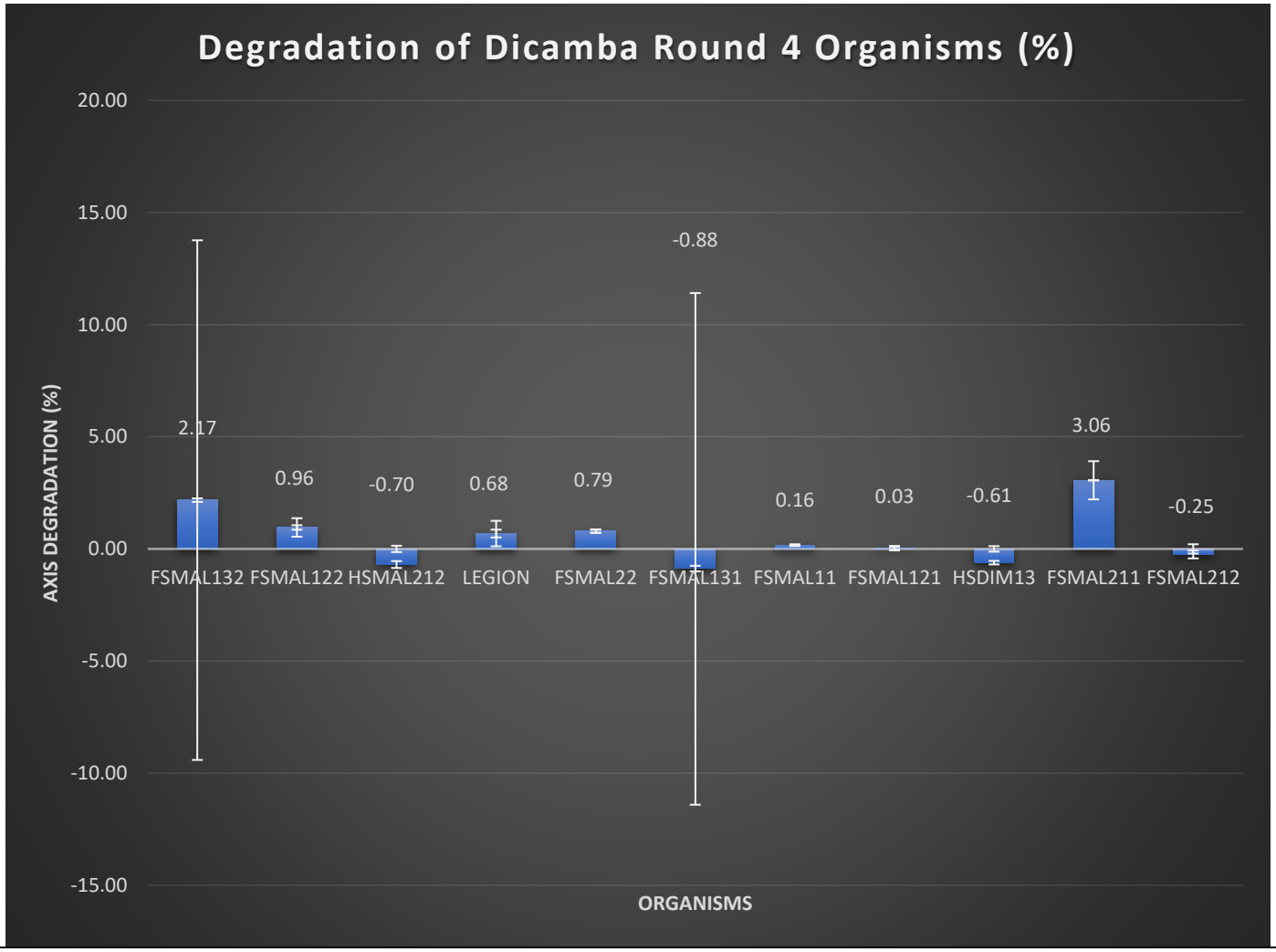

Figure 20. Degradation of Dicamba for the fourth round of organisms, in percentages. The organisms keep the same nomenclature explained above, except for LEGION-a mixed culture. Error bars represent propagated standard deviations.

3.1.6. 5th round of organisms

During the 5th round of organisms using this setup, it can be observed that the negative values have virtually been eliminated, but the degradation exhibited by most of the organisms is minimal. This round

included the 5th step of enrichment from previous work, the MMC that was to be used as base for a more efficient degrading consortium (labeled "5MMC" in the experiment). Unfortunately, the performance shown was very low with a degradation of 0.38%, well within the margin of error of the method. It also performed most poorly compared to the other cultures, where the only one with a lower degradation percentage was LOLGA. As a result, the MMC was not further utilized in degradation experiments or for enhancing other cultures.

Regarding other degraders, two of the organisms showed a degradation percentage over 3% (NOLGA and SLUDIM211), while 3 cultures showed a degradation percentage over 2% (MOLGA, SLUDIM231, HSDIC24). It seems that these 5 organisms could have pesticide degradation capabilities, but in order to further test that hypothesis, the best degraders were selected and placed on the Solid Pesticide Medium.

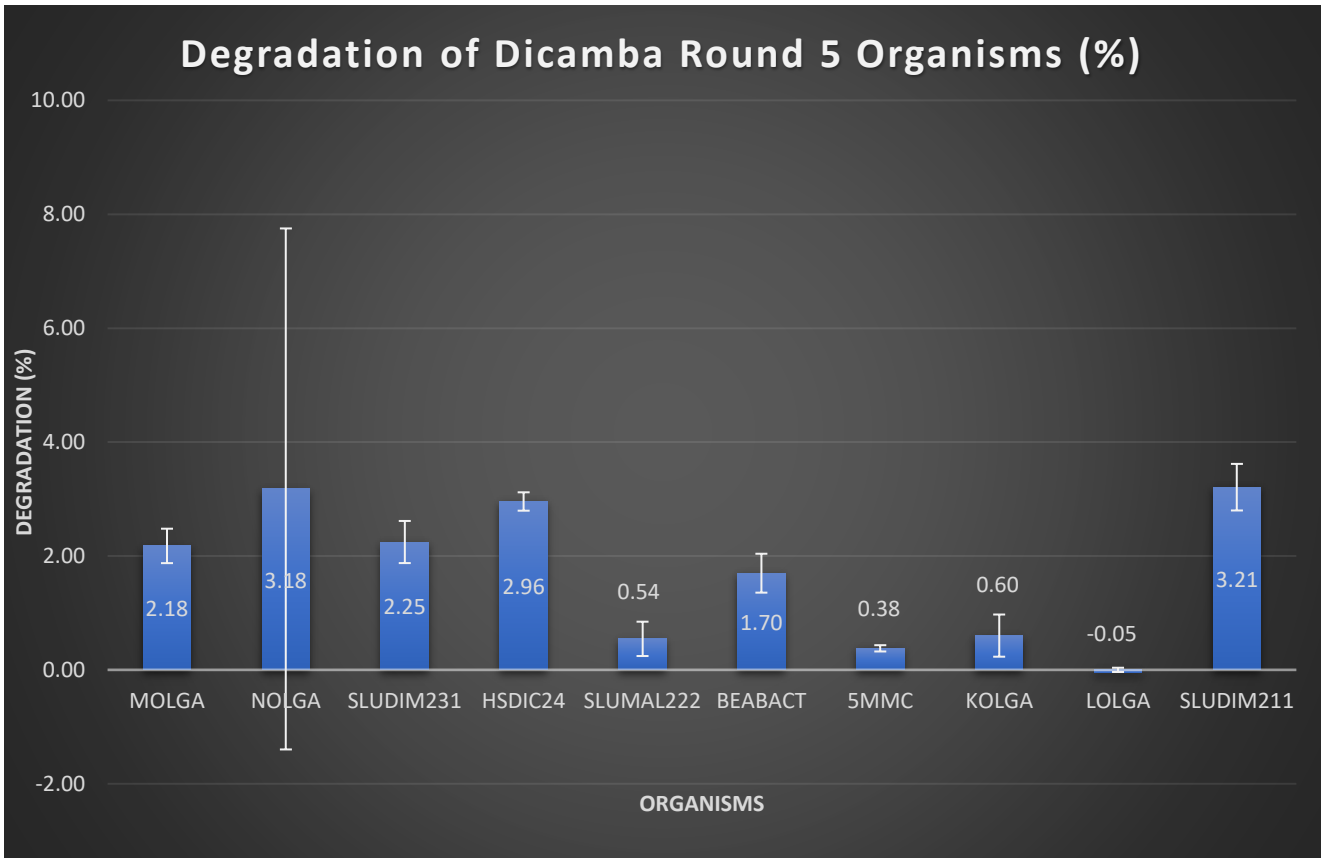

Figure 21. Degradation of Dicamba for the fifth round of organisms, in percentages. The organisms keep the same nomenclature explained above, except for BEABACT-a bacterium isolated by a colleague; 5MMC-the fifth step of enrichment on the pesticide Dicamba from previous work; MOLGA, NOLGA, KOLGA, LOLGA-*Klebsiella sp.* Error bars represent propagated standard deviations.

3.1.7. 6th round of organisms

During the 6th and final round of organisms, several organisms isolated from sludge samples (SLU) were tested. For the whole round, no organisms exceeded 2% degradation, with the highest degradation percentage being 1.86% (HSMAL12). As a result, HSMAL12 was the only bacterial isolate from round 6 that was considered for further experiments.

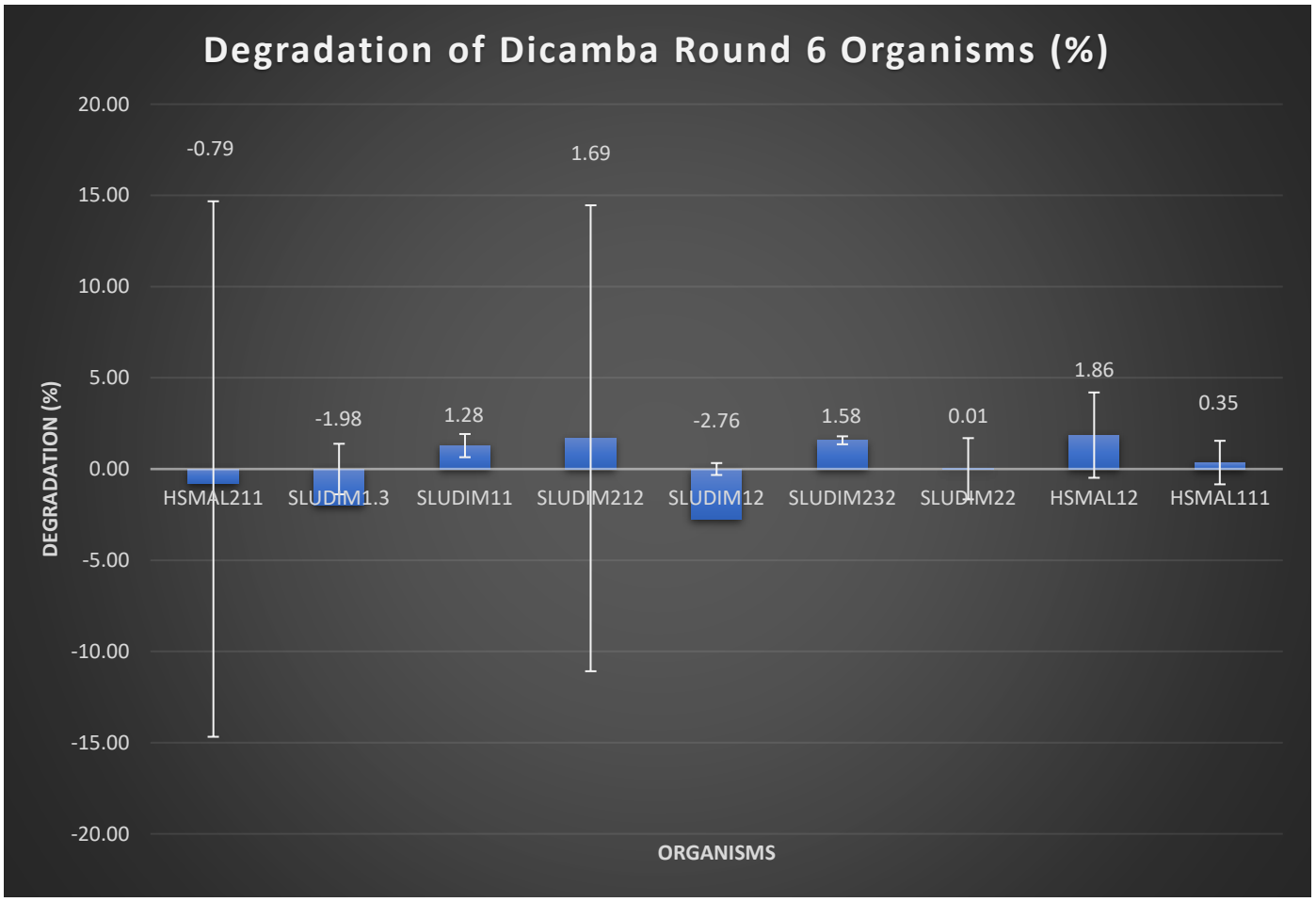

Figure 22. Degradation of Dicamba for the sixth round of organisms, in percentages. Error bars represent propagated standard deviations.

After finishing the 6 rounds, the best degraders among the organisms were selected for further experiments. Out of all the organisms tested in the Pesticide Degradation Assay, 6 bacteria were chosen as possible degraders: FSMAL211, FSMAL132, SLUDIM211, NOLGA, HSDIC24 and HSMAL12.

Firstly, they were grown on the Solid Pesticide Medium, in order to test their growth capabilities, and then they would be placed in the improved setup of the Pesticide Degradation Assay. The new setup uses 50 mL working volume instead of 20 ml, parafilm instead of cotton caps to prevent evaporation and eliminate it as a variable, and Enhanced M9 medium instead of M9 medium to further improve the growth conditions. The organisms were placed on an intermediary medium (Yeast Extract M9) for 2 days after there were grown in LB, to help with adaptations to less nutritive medium. Before being

placed in the Enhanced M9 working medium with 50 mg/L of Dicamba, the samples taken from the Yeast Extract M9 were centrifuged at 4000 rpm for 7 minutes and washed 3 times to reduce nutrients. The duration of the experiments has been increased from 2 weeks to 1 to 2 months.

The presence of LB nutritive medium in the working setup could explain the low degradation or the inability of the organisms to adapt to utilizing Dicamba as carbon source.

It has been shown that nutritive medium can disrupt the ability of organisms to degrade pesticide, while at the same time a concentration of just 50 mg/L of Dicamba as carbon source may not be enough to sustain the growth of the cells. Due to evaporation, the up concentration of the salts in the M9 medium could also provide increased osmotic pressure, that can disturb the growth and metabolic activity of the cells.

This provided grounds for improving the assay, through the additions and modifications mentioned above.

3.2. The effect of different pesticide concentrations on bacterial degradation

In order to better understand the optimal parameters of degradation setups, different concentrations of Dicamba were employed. The organism used in the experiment was HSDIC24, which appeared to be able to degrade Dicamba in the first round of the Pesticide Degradation Assay. While the Pesticide Degradation Assay utilizes only 50 mg/L of Dicamba, higher concentrations were tested in this experiment.

The original hypothesis was that if the organisms were able to tolerate the increase in Dicamba concentration, they could possibly have better growth due to the increase in carbon availability. Since Dicamba is a recalcitrant compound, the fact that increased concentrations might inhibit cell growth had to be considered as well. A graph was plotted using the data from the 3 weeks of the experiment (Fig. 23).

As it can be observed, the degradation exhibited by the 50 mg/L condition is of a low negative value, suggesting that no degradation took place, which contradicts the 5% degradation observed in the Pesticide Degradation Assay. This can be attributed to a multitude of factors, starting from the change in the vessel material and shape (in this experiment 50 mL Flacon tubes were used, which have a conical shape and are composed of plastic, while in the Pesticide Degradation Assay, 100 mL serological vials are used, which have a round base and are composed of glass). Another factor that should be considered is the amount of headspace available, as the 50 mL Falcon tubes have a smaller headspace than the 100 mL serological vials when the same working volume is used, in this case, 20 mL. These could all represent possible explanations for the difference in results.

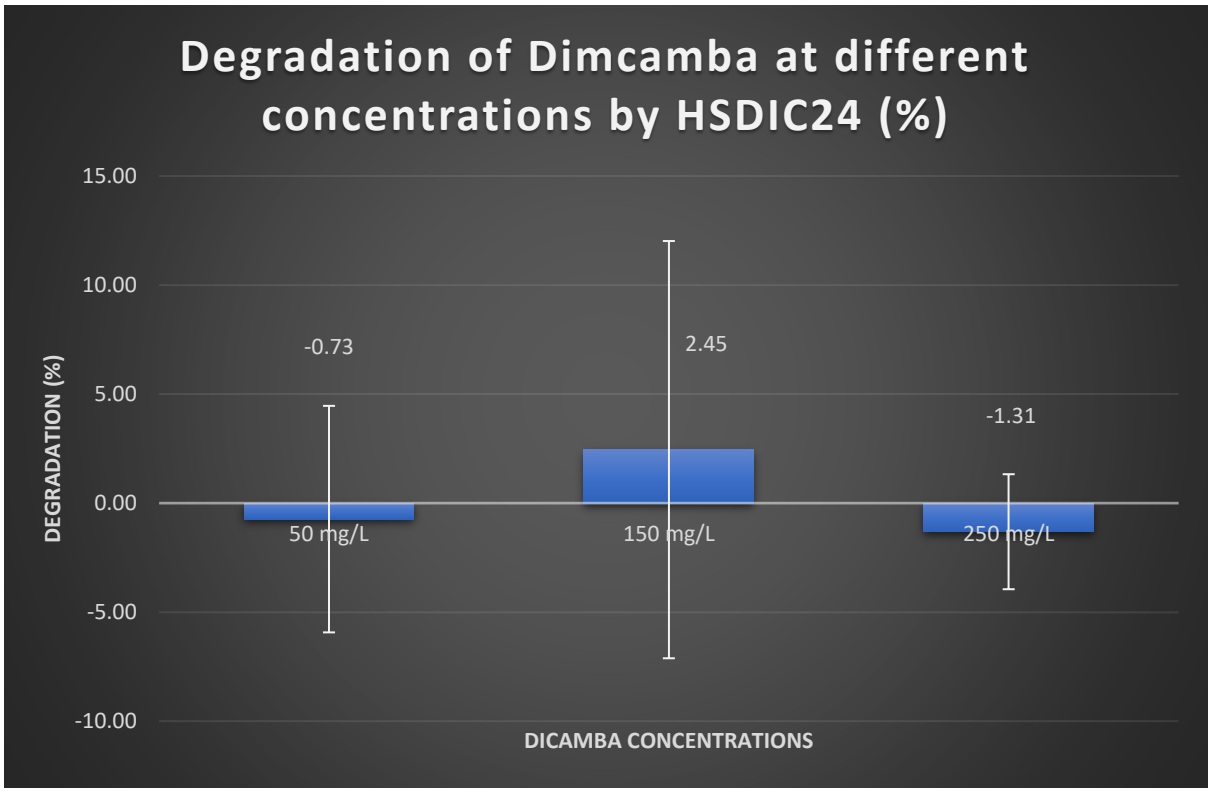

Figure 23. Degradation of Dicamba at concentrations of 50 mg/L, 150 mg/L and 250 mg/L by the possible degrader from the first round of the Pesticide Degradation Assay HSDIC24. Error bars represent propagated standard deviations.

Regarding the 150 mg/L concentration of Dicamba, a degradation percentage of 2.46% has been registered. Considering the parameters, it could mean that at 150 mg/L, by increasing the carbon source, the growth of HSDIC24 has been improved, and at the same time the concentration is not high enough to be inhibitory to the cells.

As such, it is a suitable concentration for degradation experiments in terms of available carbon, but it is a high concentration compared to what is to be found in pesticide contaminated soils.

For the 250 mg/L, the value of degradation is negative (-1.31%), which suggests that degradation of Dicamba did not take place, which can be attributed to the high pesticide concentration. A concentration of 250 mg/L could prove inhibitory to cells, and even though more carbon is available for degradation, their growth is hindered. As such, this is not a suitable concentration for the degradation experiments. Some other organisms that can degrade Dicamba found in this study might prove effective at degrading high concentrations of Dicamba, but the concentration does not match what is found in soils where Dicamba was applied or field studies.

3.3. Enrichment of soil bacteria

In the second passage, the vessels still contain a significant amount of soil from the original sample. As a result, the amount of nutrients present is rather high compared to Enhanced M9 with 50 mg/L of Dicamba, a minimal medium.

Using the Cell Viability Assay, the development of the cultures was followed. The results are expressed in million light units, and the values represent the amount of metabolically active cells present in the culture. It can be observed that there is a high degree of variability in the first measurement of passage 2, with FMC WEST having a value of 57.6 mil light units, and both FMC MP and FS having the values 19.8 and 18.5 mil light units. In the measurement after the refeeding, which involved adding 5 mL of Enhanced M9 medium with 50 mg/L of Dicamba, 3 of the 4 values increase, suggesting that the increase in carbon source provided was required, possibly due to depletion. During the last measurement, the trend remains rather constant, with FMC MP and FS having the lowest values (4.8 and 8.6 mil light units), but compared to the first measurement, now the highest value belongs to HS (33.4 mil light units), while FMC WEST registered a value of 30.3 mil light units. All the final values are smaller than the initial ones, due to depletion of the nutrients present in the soil. At the end of the measurement, 2 mL were taken from the flask and were used to inoculate another Erlenmeyer flask that contained 18 mL of Enhanced M9 medium with 50 mg/L of Dicamba.

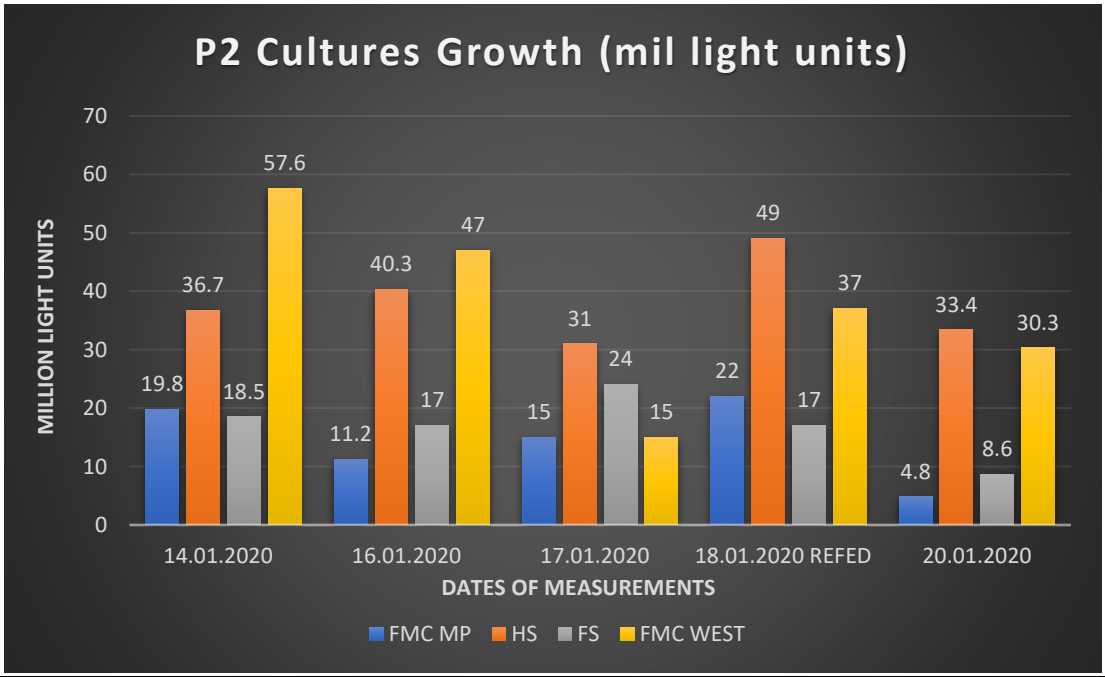

Figure 24. Microbial Cell Viability Assay measurements for Cell Passage 2; REFED-another 5 mL of Enhanced M9 with 50 mg/L of Dicamba were added to the flask after the previous measurement.

In the third passage, the values of the first measurement are much lower than in the second passage, the highest being 2.9 mil light units compared to 57.6 mil light units in the previous passage. This can be attributed to the depletion of the nutrients from the soil. For the second measurement, the values have drastically increased, with 3 of the values being between 19 and 25 mil light units (FMC MP-19.5 mil light units; FS-24 mil light units; FMMC WEST-25.83 mil light units). The 4th value, that belongs to HS, has increased to 45.32 mil light units, the highest value recorded in the experiments. Since HS showed the highest final value in P2 as well, it could mean that it is the most adapted and the fastest culture at degrading Dicamba. After the refeed step, the changes are not as significant as for the previous passage, with most values remaining very similar. While the refeed step measurement was taken after 4 days, just like in the previous passage, its impact was much smaller. This can be attributed to the lack of a secondary source of nutrients, since the soil was depleted.

The final values of P3 are between 4.4 mil light units and 8.6 mil light units, with one exception, HS, which has a value of 17.1 mil light units. In this passage, HS was recorded the highest increase in metabolically active cells, and it kept the same high values for the duration of P3. At the end of P3, 1 mL aliquots were frozen at -20 °C for further experiments, and 2 mL were taken to serve as inoculum for the next passage.

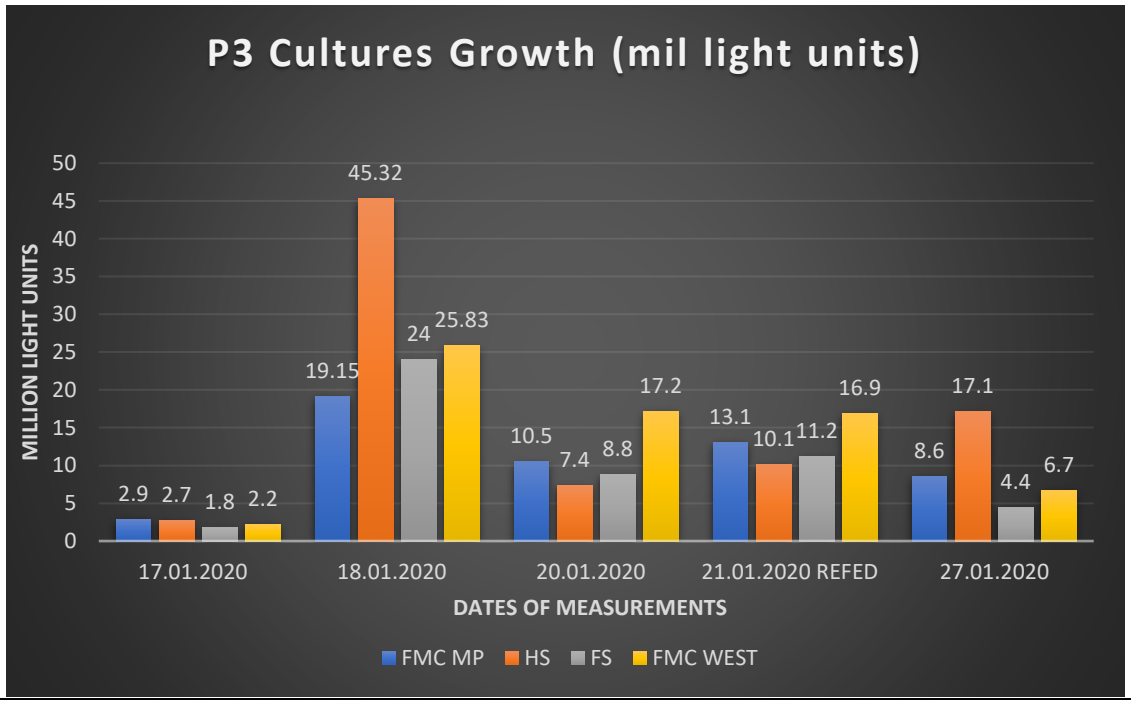

Figure 25. Microbial Cell Viability Assay measurements for Cell Passage 3; REFED-another 5 mL of Enhanced M9 with 50 mg/L of Dicamba were added to the flask after the previous measurement.

Regarding the fourth and final passage, since the cultures seem to require little intervention and since the refeed step seemed to lose its effect once the soil was washed out, only an initial and a final measurement were taken. In the initial measurement, FMC MP has the highest value, contrary to previous passages, and it retains the highest value for the 1-week duration of the passage.

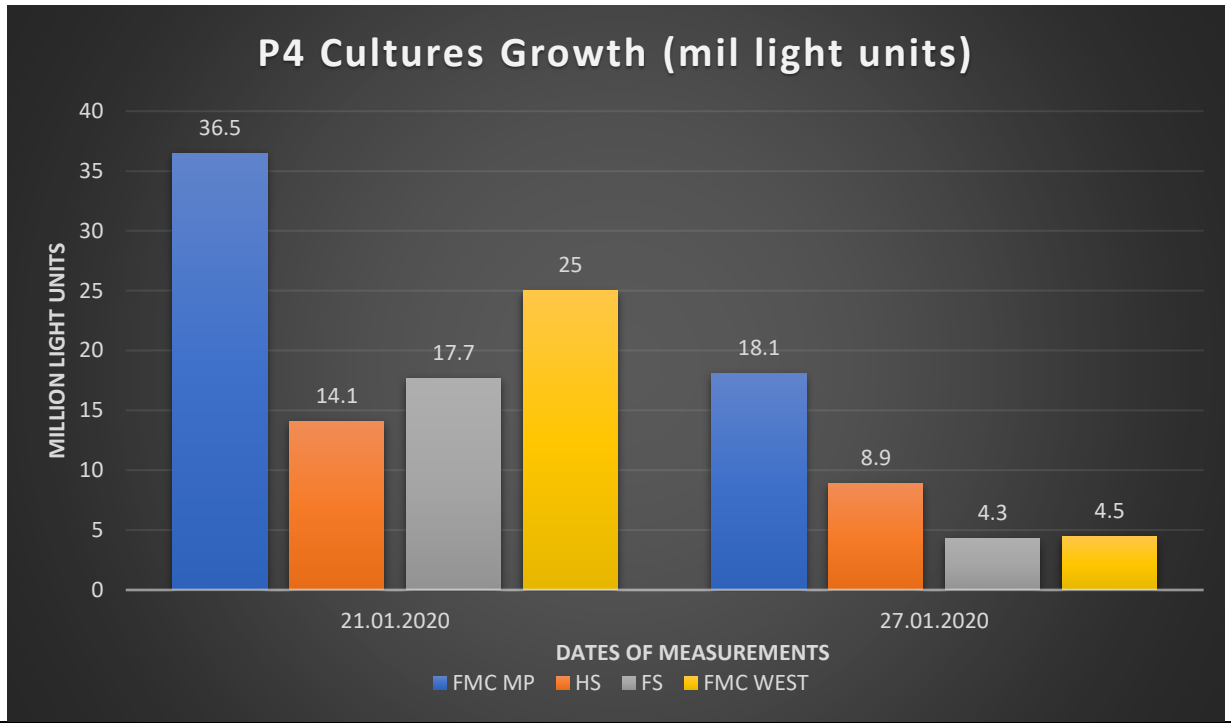

Figure 26. Microbial Cell Viability Assay measurements for Cell Passage 4.

HS, the culture that performed well in the third passage, starts with the lowest value 14.1 million light units, but after 1 week has the second highest value of the cultures (8.9 mil light units). It appears that with the depletion of the soil and lack of a secondary carbon source, the best performing culture is FMC MP, and the second one HS. FS and FMC WEST are very close in terms of values for the final passage. After the 1-week mark of the fourth passage, the four cultures were plated on Enhanced M9 Agar with 50 mg/L of Dicamba.

3.4. Growth of pesticide degrading organisms on solid medium; Recovery of the pesticide Dicamba from solid medium

From the AAU organism library, three organisms were chosen due to their potential pesticide degrading abilities: *Bacillus subtilis, Pseudomonas putida* and *Micrococcus luteus.* Some of the organisms, mainly

the *Bacillus* genus, are well-known for also having biological control function against various pathogens and difficult growth conditions, such as drought.

The negative control was represented by the same agar medium, but without Dicamba as carbon source, in order to test if the organisms are feeding on the pesticide.

After the optimization of the method, multiple organisms were tested, as this assay can provide a fast way of identifying pesticide degraders, while at the same time giving insight into the growth capabilities of each culture.

After 2 weeks of growth, the amount of Dicamba left in the agar can be quantified by freezing at -20 °C and then thawing the agar, centrifugation and the resulted supernatant can be analyzed in the HPLC.

The first organisms tested were *Bacillus subtilis* (Fig. 27), *Pseudomonas putida* (Fig. 28) and *Micrococcus luteus* (Fig. 29). The organisms showed significant growth after one week of incubation at 30°C.

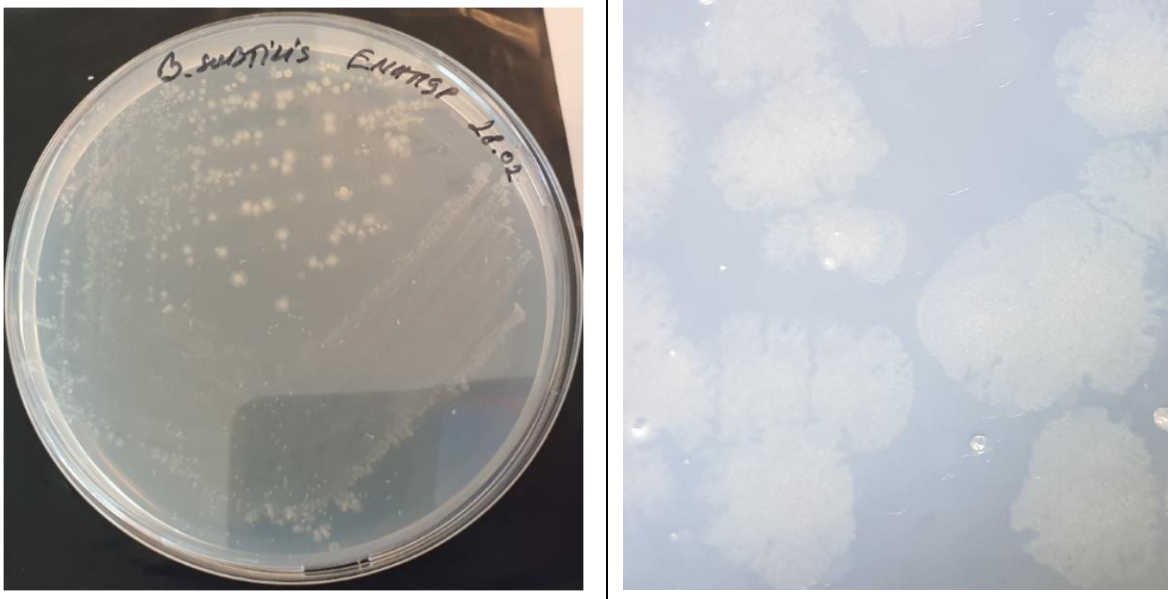

Figure 27. *Bacillus subtilis* Petri dish, Enhanced M9 Agar with 50 mg/L Dicamba after 1-week incubation at 30°C (left); Picture of the colonies taken from the stereomicroscope (right).

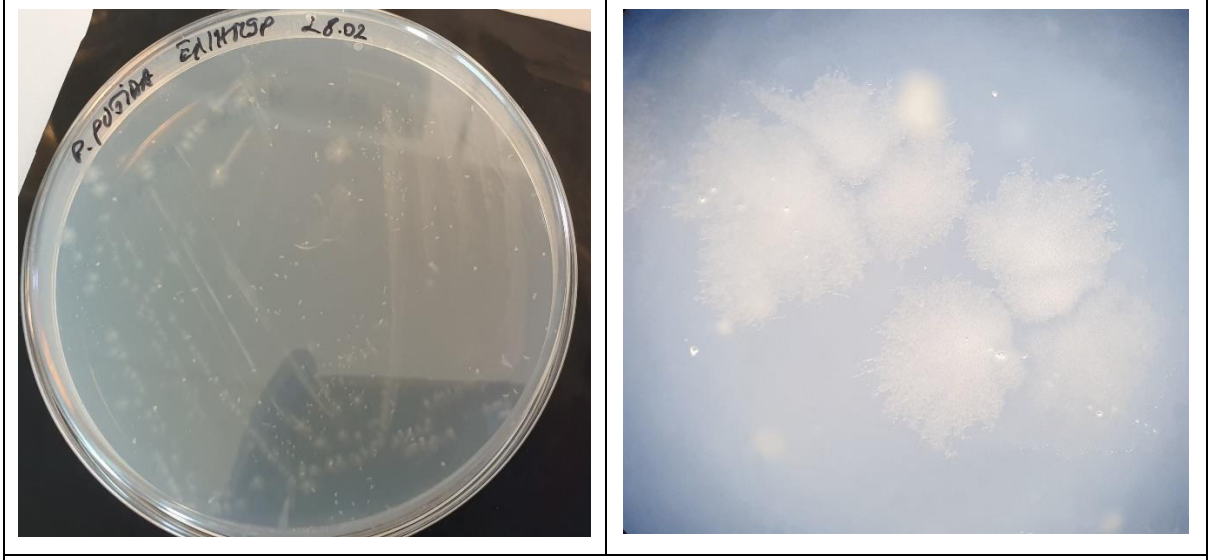

Figure 28. *Pseudomonas putida* Petri dish, Enhanced M9 Agar with 50 mg/L Dicamba after 1-week incubation at 30°C (left); Picture of the colonies taken from the stereomicroscope (right).

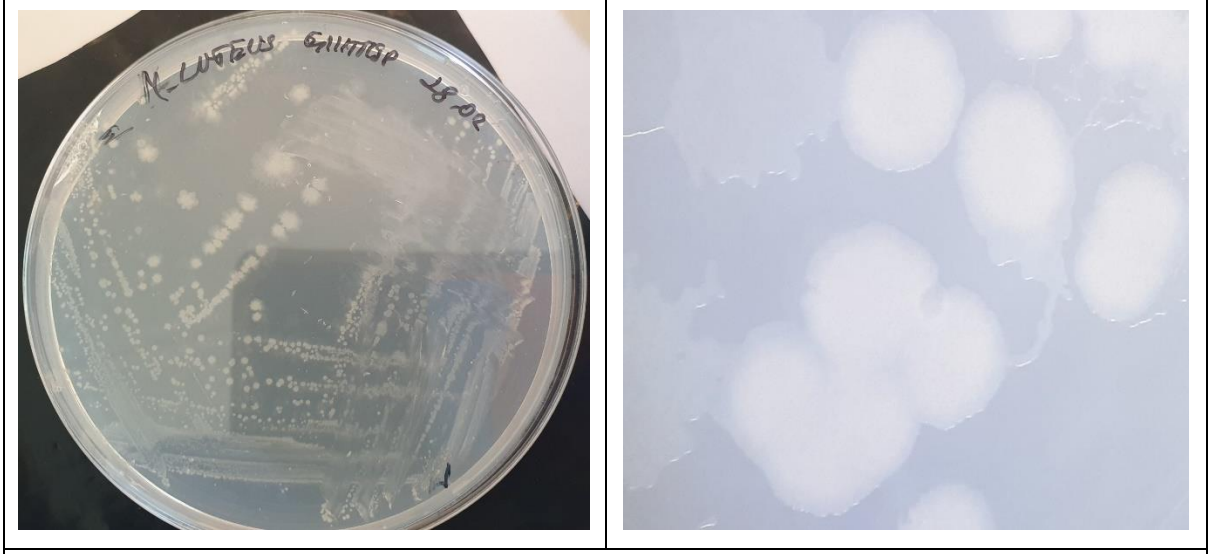

Figure 29. *Micrococcus luteus* Petri dish, Enhanced M9 Agar with 50 mg/L Dicamba after 1-week incubation at 30°C (left); Picture of the colonies taken from the stereomicroscope (right).

As it can be observed, all 3 of the organisms showed growth, and pictures were taken from the stereomicroscope in order to determine the shape and size of the colonies.

The assay is effective in selecting pesticide degrading bacteria, and since these 3 organisms seem to be able to degrade Dicamba, the plates were frozen at -20 °C in preparation for the solid quantification method of the pesticide degradation, and the organisms were also employed in the sand assay to see if they could potentially exhibit dual function, pesticide degradation and biological control against the fungal pathogen *Fusarium culmorum*.

After *Bacillus subtilis, Pseudomonas putida* and *Micrococcus luteus* were assessed, other organisms that might be able to degrade pesticide were tested as well.

Six bacterial species were chosen as possible good degraders from the Pesticide Degradation Assay: FSMAL211, FSMAL132, SLUDIM211, NOLGA, HSDIC24 and HSMAL12. Using the same protocol, they were also tested, starting with FSMAL132.

Figure 30. FSMAL132 Petri dish, Enhanced M9 Agar with 50 mg/L Dicamba after 1-week incubation at 30°C (left); Picture of the colonies taken from the stereomicroscope (right).

After 1 week, the growth of the FSMAL132 is easily observable (Fig. 30), with colonies being taken to inoculate other plates as well. Since the organism managed to grow on the solid medium with Dicamba as carbon source, it is a strong candidate for long term degradation experiments. Also, after 1 more week, this plate was also frozen at -20 °C in preparation for the solid quantification method of Dicamba degradation.

3.5. Sand assay to evaluate the efficacy of biocontrol agent wheat seed coating against seed-borne *Fusarium culmorum*

After 8 days of incubation at 22 ℃ in a growth chamber, the seedlings were assessed based on general aspect and the length of the shoot. The seedlings were watered in each replicate with 50 ml of water applied to the saucer. The seedlings were photographed and measured in order to ensure an even distribution of size. No symptoms were present at this point on any of the plants.

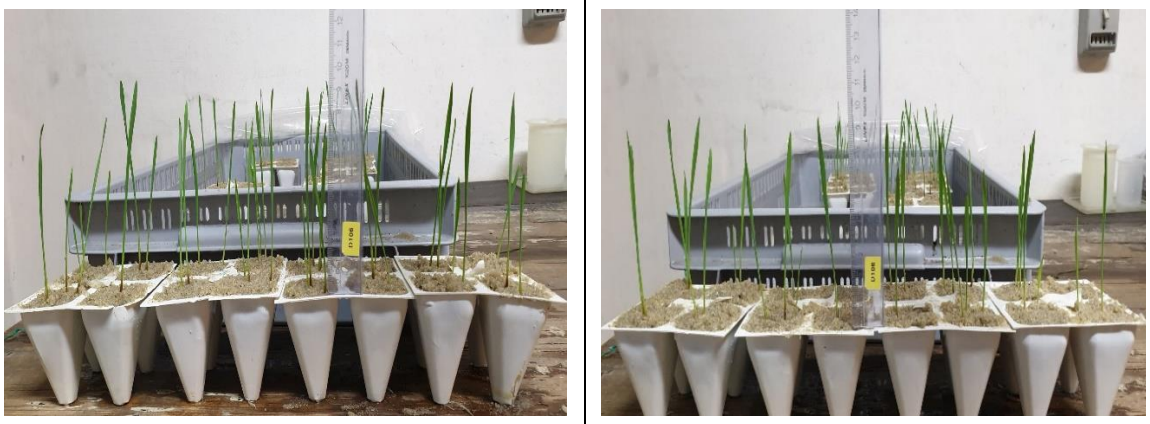

Figure 31. Wheat seedling sand assay after 8 days of incubation. Comparison between the healthy control (left) and the *Fusarium culmorum* coated seeds (right).

After 14 days from sowing the seeds, the seedlings were removed from the pots, washed, and the length and aspect of the roots and coleoptiles was assessed. The replicates were separated into blocks from 1 to 4 in order to ease their categorization.

3.5.1. *Pseudomonas putida KT2440* as biocontrol agent

The first experiment, which was also employed in order to verify the efficacy of the method, was performed using *Pseudomonas putida* KT2440 as a biocontrol agent. Species of *Pseudomonas* are known for both their effect as biocontrol agents against fungal pathogens and their ability to degrade certain pesticides (Johri, Qazi et al. 1991; Weller 2007). As a result, they were of interest to this study.

After separation into blocks and washing of seedlings, the disease index was calculated to determine the effect the biological control agent *Pseudomonas putida* had on the activity of the fungal plant pathogen *Fusarium culmorum* by assessing the severity of Fusarium Seedling Blight symptoms present.

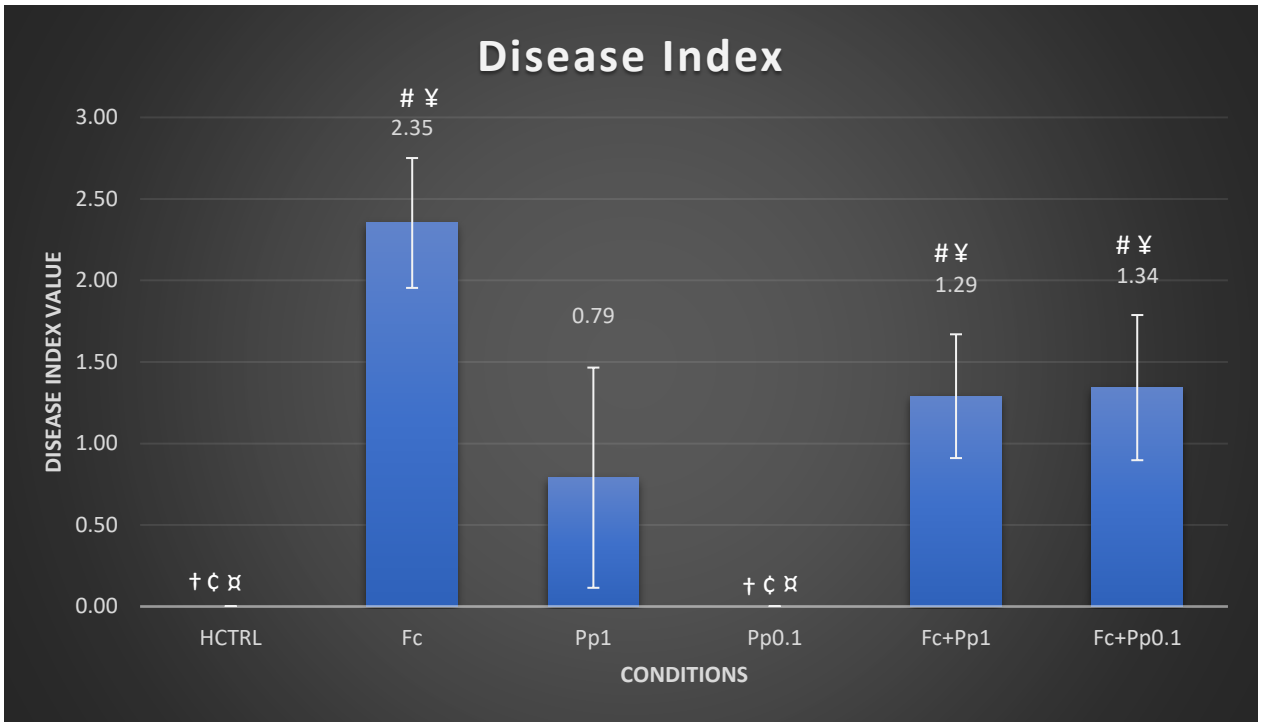

Figure 32. Biological control of Fusarium seedling blight in wheat caused by *Fusarium culmorum*. The effect of seed coating with the BCA *Pseudomonas putida* on the severity of disease symptoms. HCTRL-Healthy control; Fc-*Fusarium culmorum;* Pp1-*Pseudomonas putida* at an Optical Density of 1; Pp0.1-*Pseudomonas putida* at an Optical Density of 0.1. #-significant difference from the first condition; †-significant difference from the second condition; ‡-significant difference from the third condition; ¥-significant difference from the fourth condition; ¢-significant difference from the fifth condition; ¤-significant difference from the sixth condition; Error bars represent the Standard Error of Mean (SEM).

Using the data from the 4 blocks of this experiment, a graph was plotted. The graph shows the average values of the four blocks, with the error bars being represented by the Standard Error of Mean (SEM). Due to possible cross-contamination of the Pp1 conditions with *Fusarium culmorum*, the disease index is different from 0, with the plants exhibiting the same symptoms as the *Fusarium*-infected plants, but the difference between the *Pseudomonas putida* at an OD of 1 and the healthy control is not statistically significant.

While multiple significant differences are present, the difference between the *Fusarium culmorum* only condition and the *Fusarium culmorum* + Biological control agent (Fc+Pp1 and Fc+Pp0.1) was not statistically significant, with a p-value of 0.10 between Fc and Fc+Pp1 and 0.14, between Fc and Fc+Pp0.1. As a result, it cannot be stated that *Pseudomonas putida* KT2440 decreased the symptoms of Fusarium seedling blight.

Compared to an already established biological control agent, *Clonostachys rosea* strain IK726, used by Jensen et al in 2000 (Jensen, Knudsen et al. 2000), the trend that should have been seen becomes evident.

During the growth chamber sand test with *C. rosea*, after 19 days of incubation, the disease index was significantly lower than the *Fusarium* condition, and close to the disease index of the healthy control samples (disease index of 0.34 for fresh *C. rosea* compared to 1.61 for the *Fusarium* only condition). At the same time, plant emergence was significantly increased by all seed treatments (application of *C. rosea* IK726 and the seed protectants Sepiret and Sibutol), a trend that has not been observed during the experiments performed with *Pseudomonas putida* KT2440.

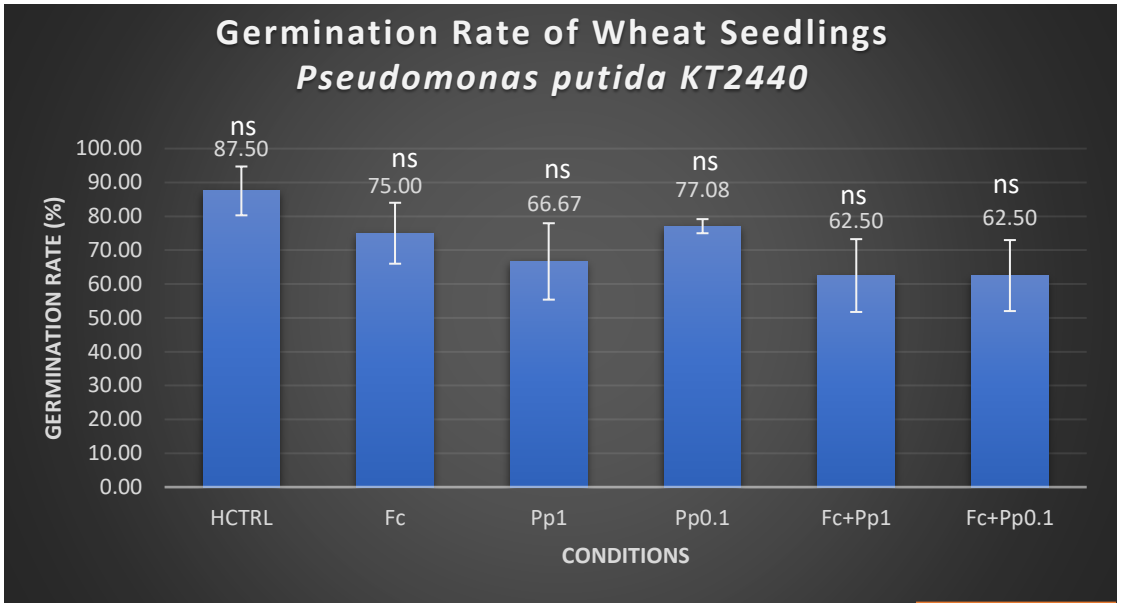

Figure 33.Biological control of Fusarium seedling blight in wheat caused by *Fusarium culmorum*. The effect of seed coating with the BCA *Pseudomonas putida* on germination rate. HCTRL-Healthy control; Fc-*Fusarium culmorum;* Pp1-*Pseudomonas putida* at an Optical Density of 1; Pp0.1-*Pseudomonas putida* at an Optical Density of 0.1. Error bars represent the Standard Error of Mean (SEM).

After determination of the disease index, the germination rate of the conditions was also tested, in order to determine if the biological control agent had any negative effects on the germination rate of the seedlings. While there is variation in the germination rate, none of the differences are statistically significant. It should also be noted that the seed lot used in this experiment had a low germination rate. As such, *Pseudomonas putida* KT2440 did not affect the germination rate of the wheat seedlings.

3.5.2. *Bacillus subtilis*, *Micrococcus luteus* and *Pseudomonas putida* as biocontrol agents

The second experiment was performed using *Bacillus subtilis*, *Micrococcus luteus* and a different strain of *Pseudomonas putida* as biocontrol agents. All the organisms were able to grow on the Enhanced M9

Agar with 50 mg/L of Dicamba, and as such if they are able to inhibit the growth of the *Fusarium culmorum*, it can be said that they exhibit dual function.

After separation into blocks and washing of seedlings, the disease index was calculated to determine the effect the biocontrol agents had on the activity of the pathogen *Fusarium culmorum* by assessing the severity of seedling blight symptoms present.

A column graph was plotted using the data from the 4 blocks. The values of the disease indexes from the four blocks were averaged, and the error bars represent the Standard Error of Mean (SEM).

The *Bacillus subtilis* strain had no effect on the symptoms of Fusarium seedling blight present. The condition that utilized *Micrococcus luteus* as biological control agent against the *Fusarium* (Fc+Ml) has a significantly lower disease index than the condition that utilized *Bacillus subtilis* as biological control agent (Fc+Bs)(statistically significant with p=0.01), but the difference between the Fc condition and Fc+Ml condition was not statistically significant (p=0.06). As a result, it cannot be stated that any of the utilized biocontrol agents decreased the Fusarium seedling blight symptoms present.

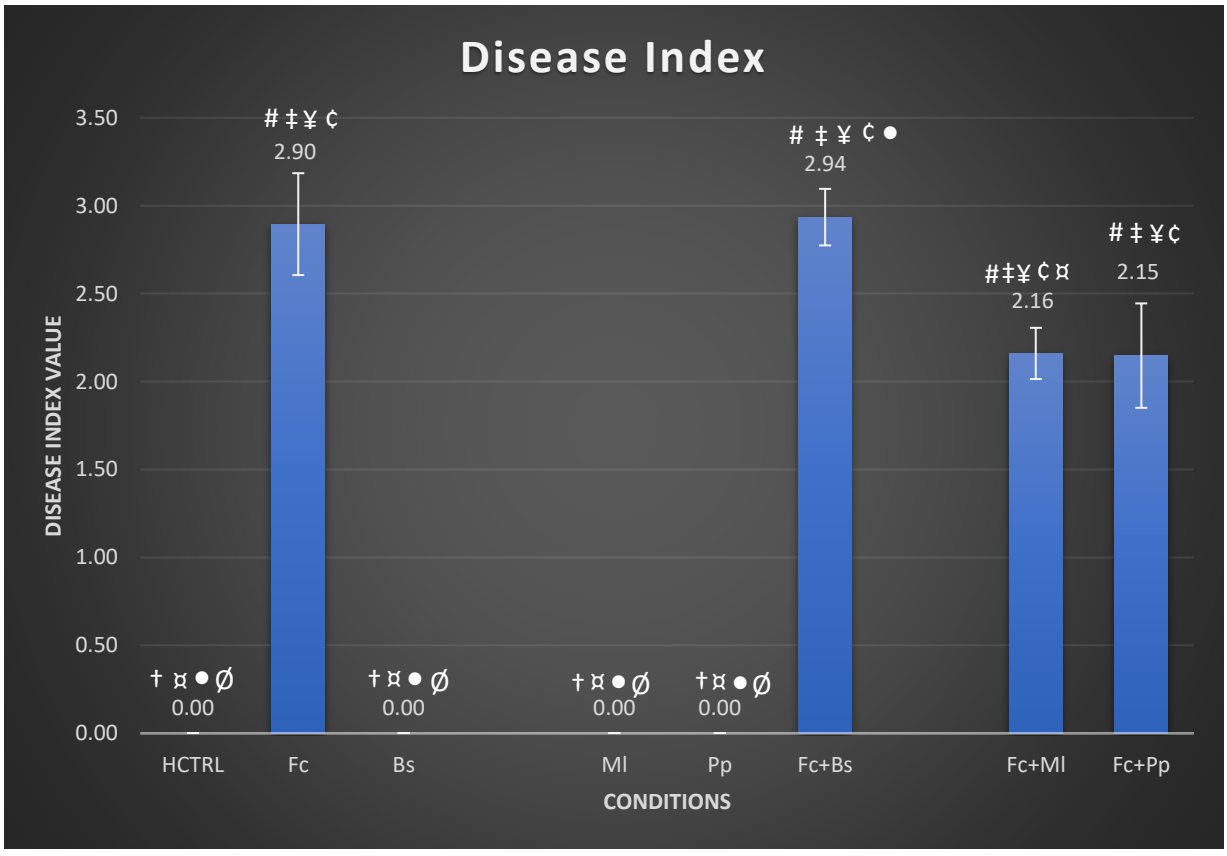

Figure 34. Biological control of Fusarium seedling blight in wheat caused by *Fusarium culmorum*. The effect of seed coating with the BCAs *Bacillus subtilis, Micrococcus luteus* and *Pseudomonas putida* on the severity of disease symptoms. HCTRL-Healthy Control; Fc-*Fusarium culmorum*, Bs-*Bacillus subtilis;* Ml-*Micrococcus luteus*; Pp-*Pseudomonas putida;* #-significant difference from the first condition; †-significant difference from the second condition; ‡-significant difference from the third condition; ¥-significant difference from the fourth condition; ¢-significant difference from the fifth

condition; ¤-significant difference from the sixth condition; ●-significant difference from the seventh condition; Ø-significant difference from the eight condition. Error bars represent the Standard Error of Mean (SEM).

Compared to the biological control agent *C. rosea*, while the disease index of the *Fusarium* only condition is higher than the one exhibited in Jensen et al 2000, conducted on winter wheat in a growth chamber (2.90 compared to 1.61), the disease indexes of all biological control agents tested in this paper remain higher than the ones exhibited by *C. rosea* under different conditions (Jensen, Knudsen et al. 2000). Inside the growth chamber, for this experiment all the conditions were incubated at 22 °C, while in the assay where *C. rosea* was utilized, a broad range of temperatures was used, 10, 15, 20 and 25 °C. Since only one temperature was tested in this assay, the results might prove different both in terms of development of the fungal pathogen and the growth of the biological control agents if different temperatures were to be tested.

Regarding the germination rate of the experiment, some differences can be observed. The *Fusarium culmorum* condition had a significantly lower germination rate compared to the control condition ($p=0.009$), but also compared to the *Bacillus subtilis* ($p=0.01$) and *Pseudomonas putida* ($p=0.01$) conditions. While the *Micrococcus luteus* conditions seems to exhibit a lower germination rate compared to the control sample, it proved not to be statistically significant ($p=0.34$). Comparing the conditions containing solely the biocontrol agents and the conditions that contain both the pathogen and the biocontrol agents, none of the bacterial species utilized as biological control agents had a negative impact on the germination rate of the wheat seedlings.

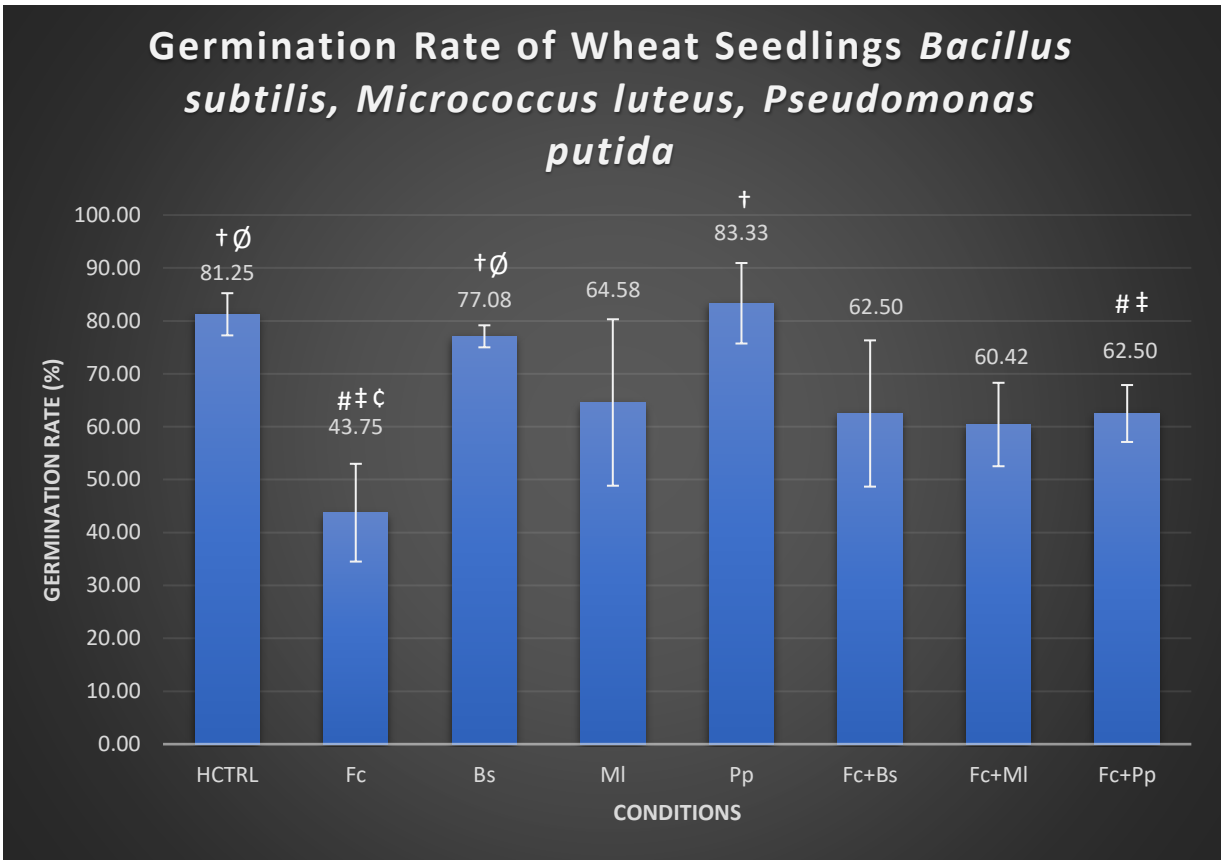

Figure 35.Biological control of Fusarium seedling blight in wheat caused by *Fusarium culmorum*. The effect of seed coating with the BCA *Pseudomonas putida* on germination rate. HCTRL-Healthy control; Fc-*Fusarium culmorum;* Pp1-*Pseudomonas putida* at an Optical Density of 1; Pp0.1-*Pseudomonas putida* at an Optical Density of 0.1. #-significant difference from the first condition; †-significant difference from the second condition; ‡-significant difference from the third condition; ¥-significant difference from the fourth condition; ¢-significant difference from the fifth condition; ¤-significant difference from the sixth condition; ●-significant difference from the seventh condition; Ø-significant difference from the eight condition; Error bars represent the Standard Error of Mean (SEM).

3.6. Sand assay to evaluate the effect of different concentrations of Dicamba on wheat plants

Since no symptoms of a specific disease are present on the seedlings, they were assessed visually based on the general aspect of the shoot and length of shoot, along with general aspect of root and size of root.

This assay was employed to determine what would be a suitable Dicamba concentration to use in further seedling assays, that would contain the fungal pathogen, different bacterial biological control agents and a pesticide concentration that would not affect the general aspect of the plant, while at the same time provide a nutrient source for the bacteria. As wheat plants are monocots and Dicamba is used to kill broadleaf weeds, which are dicots, its effects on the wheat seedlings should be minimal, but due to the fact that the application takes place on seeds instead of mature plants and that the pesticide concentrations added to the sand setup are identical to the theoretical concentration applied on the field, not the actual concentrations, as Dicamba can drift due to wind so a significant amount will not land where intended, differences can occur.

After 8 days of incubation at 22 ℃ in a growth chamber, the seedlings were assessed based on general aspect. With the increase in Dicamba concentration the length of the shoot decreases. Along with the decrease in shoot height, comes a whitening of the shoot.

After 14 days from sowing the seeds, the seedlings were washed, and the length and aspect of the roots and shoots were assessed.

Figure 36. Comparison between the Block 1 Control condition (top) and Block 1 50 mg/L Dicamba condition (bottom).

At a 50 mg/L concentration of Dicamba, the seedlings are very small compared to the control condition. The aspect of the roots is thickened, and the general length of the roots is greatly reduced. Regarding the shoots, while the control condition shoot length averages around 9 cm, the average shoot size for the 50 mg/L Dicamba condition is around 3 cm. As a result, this concentration is clearly too high to be utilized in the sand assays, as the plants are not fit for assessment.

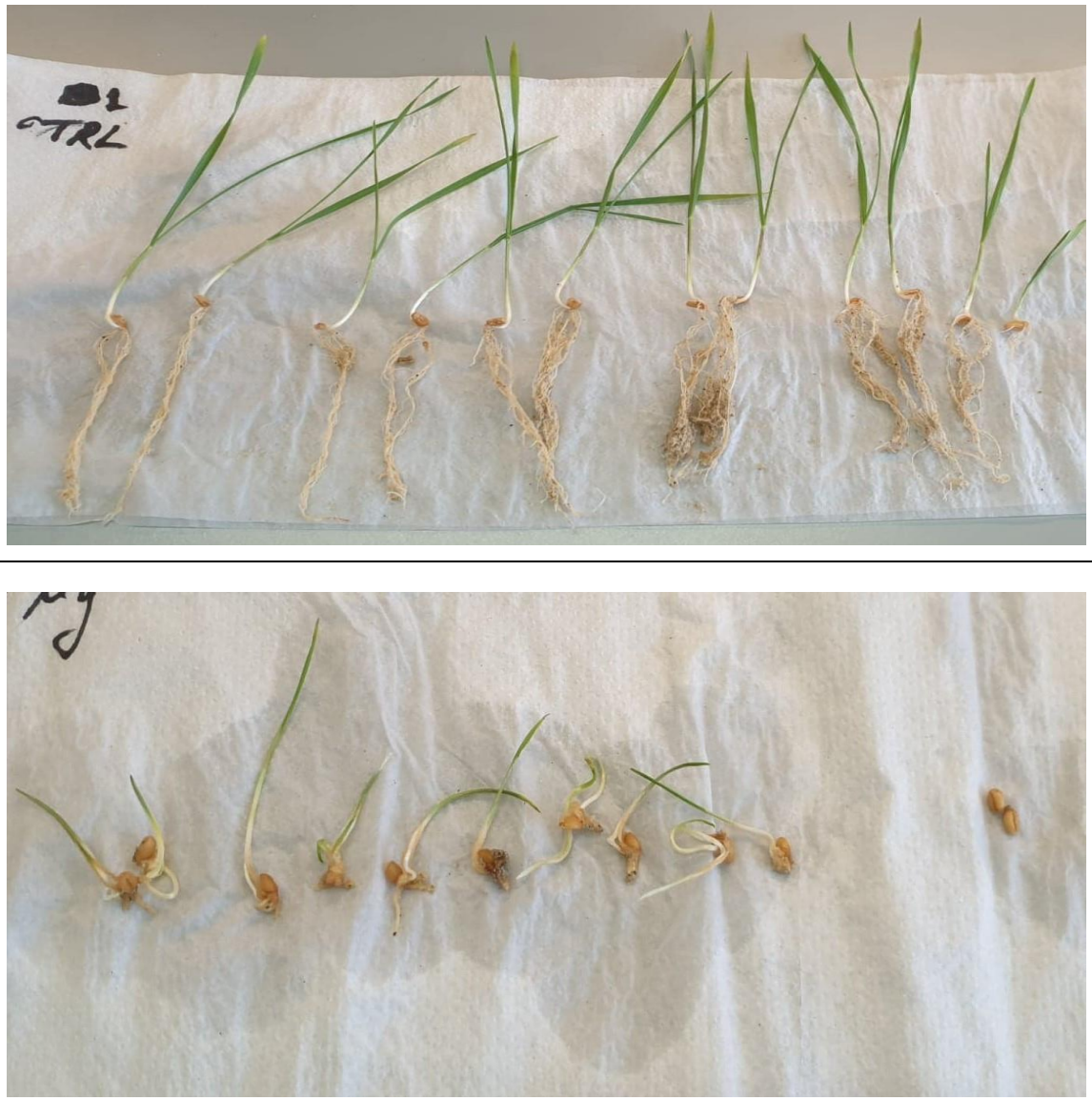

Figure 37. Comparison between the Block 1 Control condition (top) and Block 1 25 mg/L Dicamba condition (bottom).

The 25 mg/L condition is very similar to the 50 mg/L, with heavily thickened roots. The difference from the 50 mg/L condition is in the shoot length, which slightly increases, but this concentration is too high to be utilized in the sand assays as well, as it would interfere with the determination of the disease symptoms.

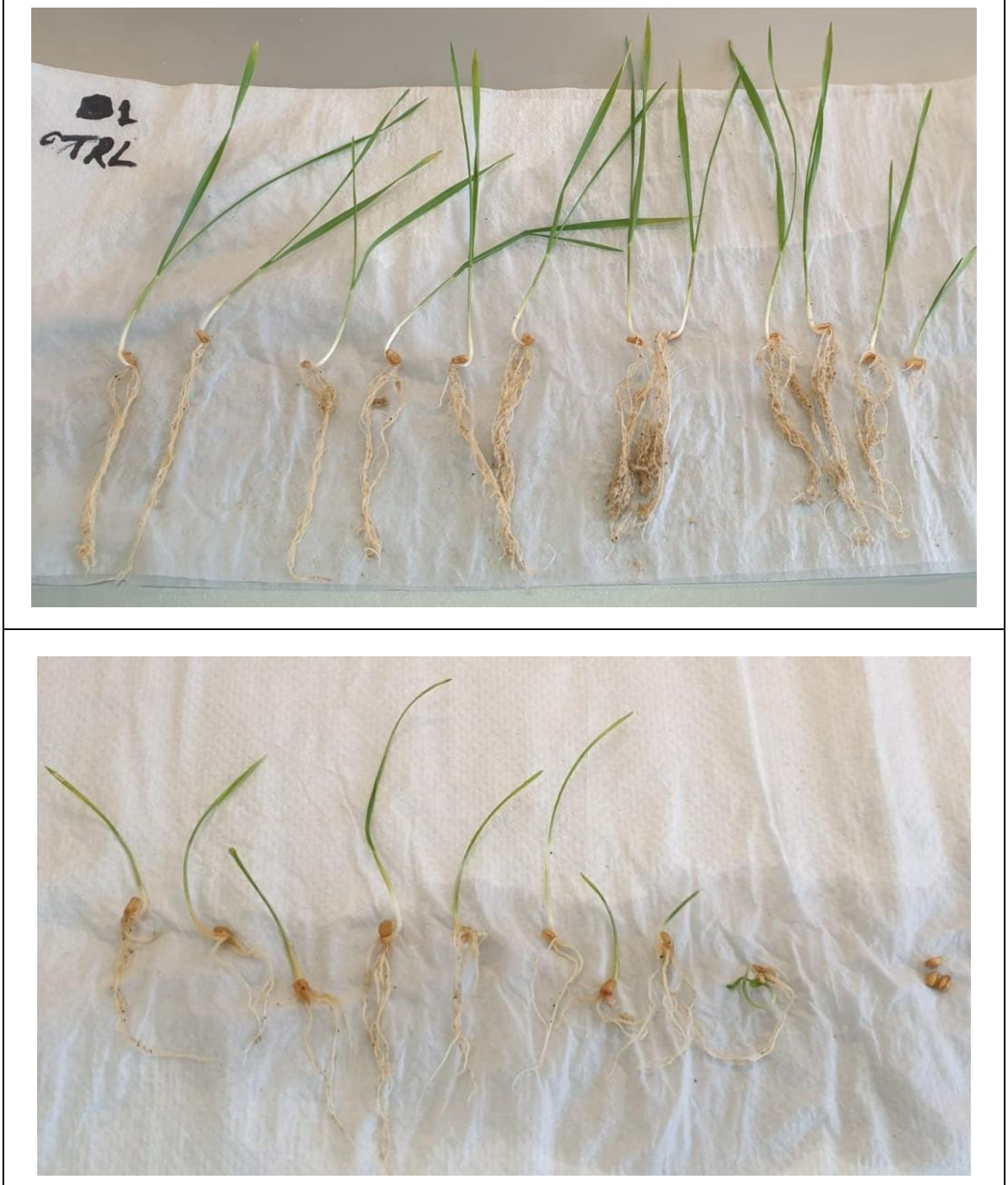

Figure 38. Comparison between the Block 1 Control condition (top) and Block 1 12.5 mg/L Dicamba condition (bottom).

Regarding the 12.5 mg/L Dicamba concentration, which also corresponds to the field conditions concentration, it can be observed that the plants are still harmed by the pesticide. While the general aspect has clearly improved compared to the 50 mg/L concentration, it is still rather different from the control group. The thickening of the roots is not very pronounced, but it still took place, and the shoot length is significantly smaller than the shoot length of the control group.

Considering the general aspect of the plants, it was determined that this concentration is also not suitable for further experiments, as the general aspect of the plants is altered, making the assessment more difficult and less reliable.

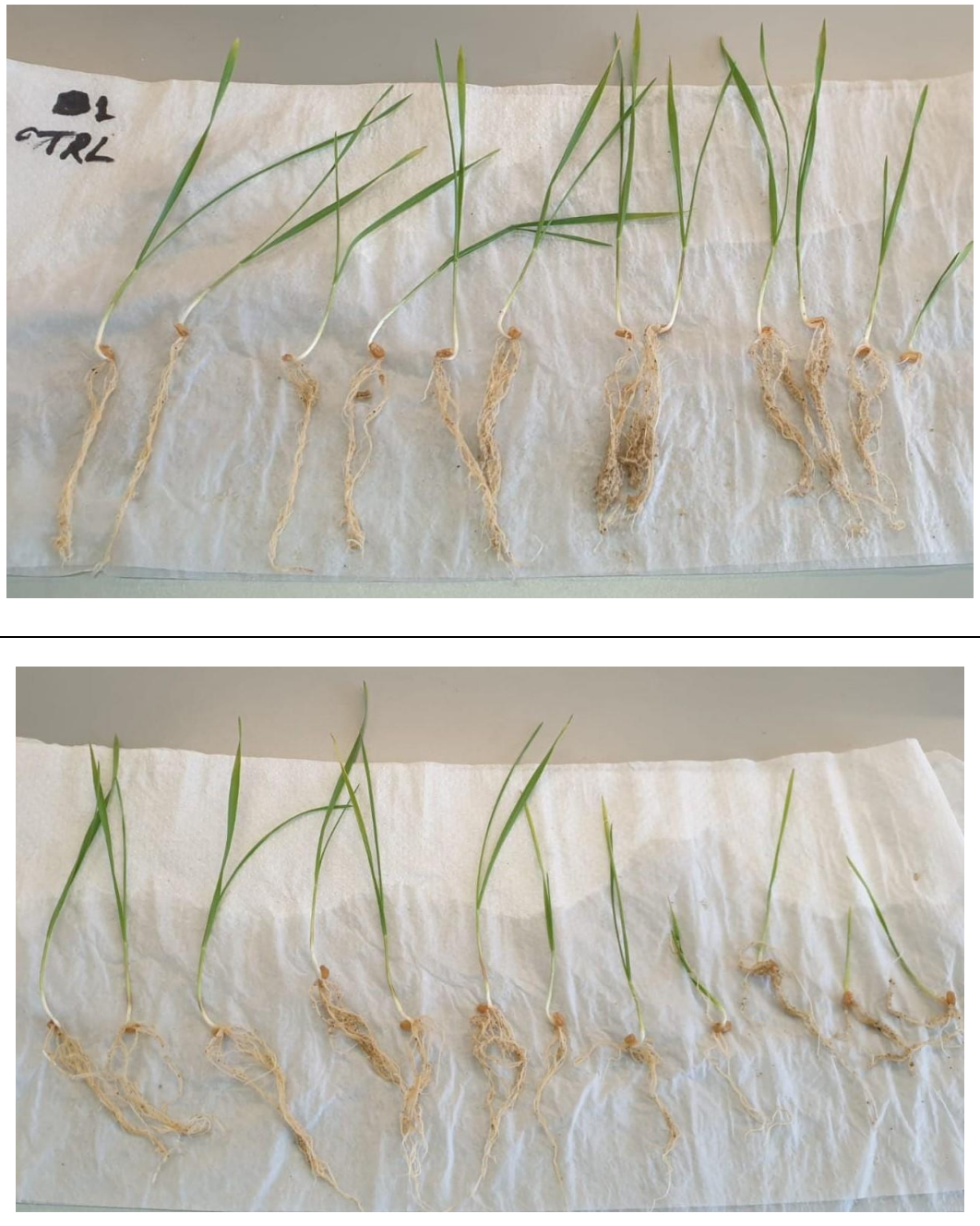

Figure 39. Comparison between the Block 1 Control condition (top) and Block 1 6.25 g/L Dicamba condition (bottom).

At a concentration of 6.25 mg/L, the effect of Dicamba on the seedlings is less pronounced. As it can be observed, a slight thickening of the roots still took place.

Regarding the shoot, the general aspect is greatly improved compared to previous, higher concentrations, the length of the shoot is similar to the length of the shoot of the control group.

While this is a low concentration of Dicamba, since it represents half of the concentration of field application, considering the drift of Dicamba, it might be more representative for the actual amount that is present on the intended crop.

This is the concentration chosen for further experiments, as it does not greatly affect the seedlings, while at the same time possible interactions with the biological control agents and *Fusarium culmorum* will determine if the pesticide has a direct effect on these organisms or acts as carbon source for the biocontrol agents and thus improves their growth and efficiency in preventing the symptoms caused by *Fusarium culmorum*.

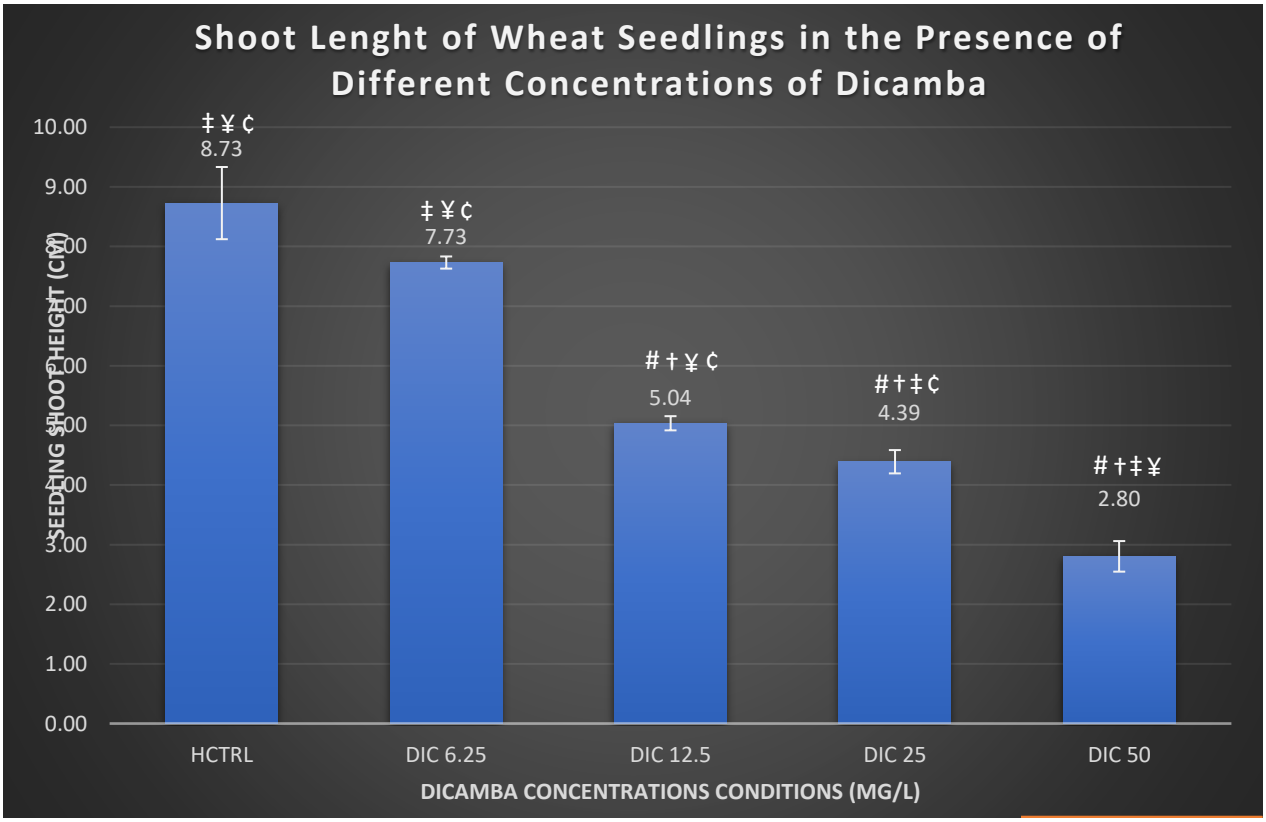

Figure 40.The effect of different concentrations of the pesticide Dicamba on the shoot length of wheat seedlings. HCTRL-Healthy control; DIC 6.25- Dicamba concentration 6.25 mg/L; DIC 12.5- Dicamba concentration 12.5 mg/L; DIC 25- Dicamba concentration 25 mg/L; DIC 50- Dicamba concentration 50 mg/L; #-significant difference from the first condition; †-significant difference from the second condition; ‡-significant difference from the third condition; ¥-significant difference from the fourth condition; ¢-significant difference from the fifth condition; Error bars represent the Standard Error of Mean (SEM).

For the quantitative data, measurements were taken of all the plants in the 3 replicate blocks and a graph was plotted. As it can be observed from the visual determination, the healthy control had an average length of 8.73 cm. The condition with only 6.25 mg/L of Dicamba had an average length of 7.73, similar

to the one of the healthy control. With the increase of the concentration of Dicamba, the length of the shoot decreases significantly(between DIC6.25 and DIC12.5, $p=0.2*10^{7}$; between DIC12.5 and DIC25, p=0.004; between DIC25 and DIC50, p=0.0005).

As mentioned above, the quantitative data confirms the visual assessment. The groups ranging from 12.5 to 50 mg/L were very badly afflicted by Dicamba, with the 6.25 mg/L only being slightly under the average height of the control group. The difference between the control group and the 6.25 mg/L of Dicamba is in the thickening of the roots, which might interfere with the physiology of the seedlings. As a result, out of the concentrations listed, the concentration that would exhibit the least amount of damage and would probably allow for assessment through the Fusarium seedling blight sand assay is 6.25 mg/L of Dicamba.

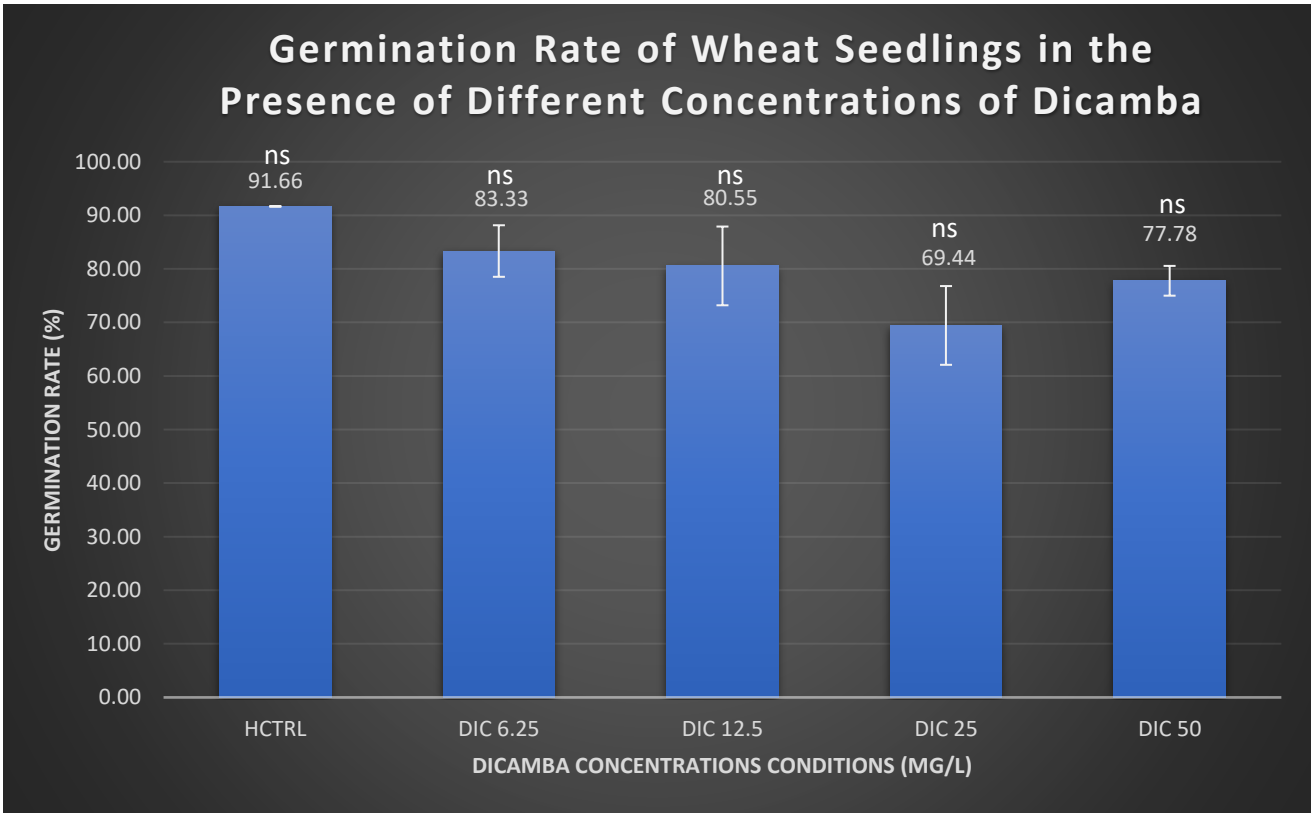

Figure 41.The effect of different concentrations of the pesticide Dicamba on the germination rate of wheat seedlings. HCTRL-Healthy control; DIC 6.25- Dicamba concentration 6.25 mg/L; DIC 12.5- Dicamba concentration 12.5 mg/L; DIC 25- Dicamba concentration 25 mg/L; DIC 50- Dicamba concentration 50 mg/L; ns- not significant. Error bars represent the Standard Error of Mean (SEM).

While the seedlings' root and shoot are affected by Dicamba, it appears that Dicamba does not have an effect on the germination rate. As it can be observed from the above figure, there is variation in the germination rate, but it is not significant.

4. CONCLUSION AND FUTURE PERSPECTIVES

The Pesticide Degradation Assay method was constantly improved for the duration of the experiments, learning from the previous rounds and changing parameters in order to minimize the error and increase degradation. Multiple sources of error have been found, such as improper solubilization of Dicamba in the working medium. If the working volume was added to the serological vials before proper solubilization of Dicamba in the 1L medium bottle, that would lead to different concentrations being present in the replicates, which could be observed in the initial rounds of the Pesticide Degradation Assay.

Another parameter which greatly influenced the accuracy of the results was evaporation. In order to determine the concentration of Dicamba present in the vials at the end of the experiments, the volume was measured, and the formula mentioned in Section 3.1.2. was employed to account for evaporation. Unfortunately, the presence of evaporation as a variable decreases the overall accuracy, even though the volume was measured at the end of the experiments, droplets of medium could still be present on the vials, which would mean that the determined volume would not be entirely correct.

In order to be able to disregard evaporation as a variable, parafilm can be used to cover the serological vials instead of the cotton caps. The properties of the parafilm allow air to pass, keeping aerobic conditions inside the vials, while at the same time preventing the loss of moisture. This would increase the accuracy of the results of the Pesticide Degradation Assay.

Along with replacing the cotton caps with parafilm, increasing the working volume from 20 mL to 50 mL would lead to a decrease in the error, since sampling would not affect the overall volume to such a large extent. Since the degradation measured after the 2 weeks of the experiment was small, another way to improve the assay would be to increase the duration of the experiments from 2 weeks to 1 month and then possibly to 2 months, depending on the degradation capabilities of the organisms.

Due to unforeseen circumstances that led to the closure of the laboratory, further testing has been impaired. If access was permitted, multiple experiments would have been performed. Firstly, the improved Pesticide Degradation Assay would have been used on the four degraders that were found, and the other untested cultures, such as the 4 MMCs obtained from contaminated soil samples (FMC MC, FS, HS and FMC WEST). During the experiments with the Enhanced M9 Agar, even though the medium nutritional content is minimal, some of the organisms thrived. As a result, since most of the organisms tested originate from soil, it could be assumed that their growth parameters would improve on solid medium. Two experiments could be performed to test the theory. The first experiment would involve growing the organisms on the Enhanced M9 Agar with 50 mg/L of Dicamba and quantifying the amount of Dicamba left at the end of the experiments. While an assay to quantify Dicamba from solid medium was devised, due to closure of the laboratory the recovery rate of Dicamba could not be

tested, nor could the samples. The second experiment would involve growing the organisms in a liquid medium, but with an inert substrate that they can attach to, such as plastic powder or glass beads to determine if the substrate would increase the degradation.

After the 6 rounds of organisms were tested, six degraders were found that can then be used in the improved setup for the Pesticide Degradation Assay and also tested using the Enhanced M9 Agar with 50 mg/L of Dicamba.

Regarding the seedling assays, multiple organisms that could possess biological control abilities against the fungal plant pathogen *Fusarium culmorum* were tested. Out of the tested organisms, *Pseudomonas putida* KT2440 and *Micrococcus luteus* seemed to be able to interfere with the growth of the fungal pathogen, but the results were not statistically significant. The assays should be repeated for these organisms. At the same time, in other papers that utilized the sand seedling assay, a broad range of temperature conditions was used, which could potentially influence the results obtained. As a result, the experiments should also be repeated with different temperature conditions and the differences should be observed.

The dual plate setup that was designed remains to be tested, as it can provide information of how the presence of the pesticide Dicamba can influence the biocontrol function of certain bacterial species against the fungal pathogen *in vitro.*

During the sand assay with application of Dicamba, concentrations above and below the field application rate were tested, with only 6.25 mg/L of Dicamba being mild enough to allow proper development of the wheat seedlings. The concentration of 6.25 mg/L represents half of the concentration used in field conditions, but it should be noted that in this experiment Dicamba is applied on seeds, and that the experiments take place in sand instead of soil, conditions that might interfere with the tolerance of the plants towards Dicamba. The concentration of 6.25 mg/L is low enough to allow proper development of the seedlings, and as a result it could be included in the *Fusarium* seedling blight assays, to simulate how the biological control agents would perform in the presence of Dicamba and how the fungal pathogen might react.

The purpose of this study was to find organisms that can accomplish two things: degrade the pesticide Dicamba and at the same time provide biological control against *Fusarium culmorum*. Unfortunately, only a few of the organisms that were tested for pesticide degradation were tested in the seedling assay as well, due to the closure of the university, and many more remain to be tested.

In conclusion, *Bacillus subtilis, Pseudomonas putida, Micrococcus luteus* and FSMAL132 were able to degrade the pesticide Dicamba, demonstrated through growing on the Enhanced M9 Agar with 50 mg/L of Dicamba, though the degraded amount remains unquantified, but did not present biological control abilities against the fungal pathogen *Fusarium culmorum.*

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APPENDIX 1

Disease index calculations for the 4 blocks using *Pseudomonas putida KT2440* as biological control agent

BLOCK 1

Table 1A. Disease index for Block 1 with *Pseudomonas putida* as biocontrol agent. HCTRL-Healthy control; Fc-*Fusarium culmorum;* Pp1-*Pseudomonas putida* at an Optical Density of 1; Pp0.1-*Pseudomonas putida* at an Optical Density of 0.1.

Treatment	Disease score (number of plants per score)					Number of scored plants (including seed killed by Fusarium)	Non-germinated healthy seed	Disease Index (DI)	Germination Rate
	0	**1**	**2**	**3**	**4**				
HCTRL	12					12	0	0	100%
Fc		2	4	2	4*	12	0	2.75	66.66%
Pp 1		4			6*	10	2	2.8	33.33%
Pp 0.1	10					10	2	0	83.33%
Fc+ Pp 1	10				1*	11	1	0.36	83.33%
Fc+ Pp 0.1	7			1	3*	11	1	1.36	66.66%

BLOCK 2

Table 2A. Disease index for Block 2 with *Pseudomonas putida* as biocontrol agent. HCTRL-Healthy control; Fc-*Fusarium culmorum;* Pp1-*Pseudomonas putida* at an Optical Density of 1; Pp0.1-*Pseudomonas putida* at an Optical Density of 0.1.

Treatment	Disease score (number of plants per score)					Number of scored plants (including seed killed by Fusarium)	Non-germinated healthy seed	Disease Index (DI)	Germination Rate
	0	**1**	**2**	**3**	**4**				
HCTRL	9					9	3	0	75%

Fc	3	2		2	5*	12	0	2.33	58.33%
Pp 1	9					9	3	0	75%
Pp 0.1	9					9	3	0	75%
Fc+ Pp 1	4	2			6*	12	0	2.16	50%
Fc+ Pp 0.1	9	1				10	2	0.1	83.33%

BLOCK 3

Table 3A. Disease index for Block 3 with *Pseudomonas putida* as biocontrol agent. HCTRL-Healthy control; Fc-*Fusarium culmorum;* Pp1-*Pseudomonas putida* at an Optical Density of 1; Pp0.1-*Pseudomonas putida* at an Optical Density of 0.1.

Treatment	Disease score (number of plants per score)					Number of scored plants (including seed killed by Fusarium)	Non-germinated healthy seed	Disease Index (DI)	Germination Rate
	0	**1**	**2**	**3**	**4**				
HCTRL	12					12	0	0	100%
Fc		3		2	7	12	0	3.08	100%
Pp 1	9					9	3	0	75%
Pp 0.1	9					9	3	0	75%
Fc+ Pp 1	8				3*	11	1	1.09	66.66%
Fc+ Pp 0.1	2	2			8*	12	0	2.16	33.33%

BLOCK 4

Table 4A. Disease index for Block 4 with *Pseudomonas putida* as biocontrol agent. HCTRL-Healthy control; Fc-*Fusarium culmorum;* Pp1-*Pseudomonas putida* at an Optical Density of 1; Pp0.1-*Pseudomonas putida* at an Optical Density of 0.1.

Treatment	Disease score (number of plants per score)	Number of scored plants	Non-germinated	Disease Index (DI)	Germination Rate

	0	**1**	**2**	**3**	**4**	(including seed killed by Fusarium)	healthy seed		
HCTRL	9					9	3	0	75%
Fc	6	3			3*	12	0	1.25	75%
Pp 1	10				1*	11	1	0.36	83.33%
Pp 0.1	9					9	3	0	75%
Fc+ Pp 1	5		1		3*	9	3	1.55	50%
Fc+ Pp 0.1	5	2	1		4*	12	0	1.75	66.66%

APPENDIX 2

Disease index calculations for the 4 blocks using *Bacillus subtilis, Micrococcus luteus* and *Pseudomonas putida* as biological control agents.

BLOCK 1

Table 5A. Disease index for Block 1 with *Bacillus subtilis, Micrococcus luteus* and *Pseudomonas putida* as biocontrol agents. HCTRL-Healthy control; Fc-*Fusarium culmorum;* Bs-*Bacillus subtilis;* Ml-*Micrococcus luteus;* Pp-*Pseudomonas putida.*

Treatment	Disease score (number of plants per score)					Number of scored plants (including seed killed by Fusarium)	Non-germinated healthy seed	Disease Index (DI)	Germination Rate
	0	**1**	**2**	**3**	**4**				
HCTRL	10					10	2	0	83.33%
Fc		4	3		5*	12	0	2.5	58.33%
Bs	9					9	3	0	75%
Ml	4					4	8	0	33.33%
Pp	9					9	3	0	75%
Fc+ Bs		3			9*	12	0	3.25	100%
Fc+ Ml		3	2	1	3*	9	3	1.83	50%
Fc+ Pp		5	2	1	1*	10	2	1.33	75%

BLOCK 2

Table 6A. Disease index for Block 2 with *Bacillus subtilis, Micrococcus luteus* and *Pseudomonas putida* as biocontrol agents. HCTRL-Healthy control; Fc-*Fusarium culmorum;* Bs-*Bacillus subtilis;* Ml-*Micrococcus luteus;* Pp-*Pseudomonas putida.*

Treatment	Disease score (number of plants per score)					Number of scored plants (including seed killed by Fusarium)	Non-germinated healthy seed	Disease Index (DI)	Germination Rate
	0	**1**	**2**	**3**	**4**				
HCTRL	9					9	3	0	75%
Fc		5	1		6*	12	0	2.58	50%
Bs	9					9	3	0	75%
Ml	5					5	7	0	41.66%
Pp	8					8	4	0	66.66%
Fc+ Bs		5			7*	12	0	2.75	41.66%
Fc+ Ml		4	2		4*	10	2	2.4	50%
Fc+ Pp		3	4	1	4*	12	0	2.5	66.66%

BLOCK 3

Table 7A. Disease index for Block 3 with *Bacillus subtilis, Micrococcus luteus* and *Pseudomonas putida* as biocontrol agents. HCTRL-Healthy control; Fc-*Fusarium culmorum;* Bs-*Bacillus subtilis*; Ml-*Micrococcus luteus;* Pp-*Pseudomonas putida.*

Treatment	Disease score (number of plants per score)					Number of scored plants (including seed killed by Fusarium)	Non-germinated healthy seed	Disease Index (DI)	Germination Rate
	0	**1**	**2**	**3**	**4**				
HCTRL	11					11	1	0	91.66%
Fc	1				1+10*	12	0	3.75	16.66%
Bs	10					10	2	0	83.33%

Ml	11					11	1	0	91.66%
Pp	12					12	0	0	100%
Fc+ Bs		2	1	2	7*	12	0	3.16	41.66%
Fc+ Ml	1	5	2		5*	12	0	2.41	58.33%
Fc+ Pp		5	2		3*	10	2	2.1	58.33%

BLOCK 4

Table 8A. Disease index for Block 4 with *Bacillus subtilis, Micrococcus luteus* and *Pseudomonas putida* as biocontrol agents. HCTRL-Healthy control; Fc-*Fusarium culmorum;* Bs-*Bacillus subtilis*; Ml-*Micrococcus luteus;* Pp-*Pseudomonas putida.*

Treatment	Disease score (number of plants per score)					Number of scored plants (including seed killed by Fusarium)	Non-germinated healthy seed	Disease Index (DI)	Germination Rate
	0	**1**	**2**	**3**	**4**				
HCTRL	9					9	3	0	75%
Fc		4	1	1	6*	12	0	2.75	50%
Bs	9					9	3	0	75%
Ml	11					11	1	0	91.66%
Pp	11					11	1	0	91.66%
Fc+ Bs		4	1	3	4*	12	0	2.58	66.66%
Fc+ Ml		6	3		1+2*	12	0	2	83.33%
Fc+ Pp		5		1	6*	12	0	2.66	50%