REMTECH EXPO

Bioremediation of the herbicide Dicamba associated with biological control: a possible approach

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OUTLOOK

- 1. INTRODUCTION
- PESTICIDES
- DICAMBA
- BIOREMEDIATION
- SCIENTIFIC QUESTION AND APPROACH
- EXPERIMENTAL SETUP
- 2. RESULTS
- PESTICIDE DEGRADATION ASSAY
- MEDIUM OPTIMIZATION
- ENRICHMENT PERFORMED ON SOIL SAMPLES
- PLATE ASSAY AND BACTERIAL GROWTH
- FUSARIUM SEEDLING ASSAY
- DICAMBA APPLICATION
- 3. FUTURE PERSPECTIVES AND CONCLUSION
- FUTURE PERSPECTIVES
- CONCLUSION
- ACKNOWLEDGEMENTS

INTRODUCTION: PESTICIDES

What is a pesticide ?

- Pesticides are chemical compounds that are used to prevent, destroy, repel, or mitigate pests, which include insects, rodents, fungi and unwanted plants (weeds).
- Since the beginning of agriculture, pesticides have been in use: from the use of sulphur compounds to control mites and insects in ancient times (2500 BC) to the development of modern pesticides, such as DDT (1940).

Benefits vs Risks ?

Benefits and risks associated with the use of pesticides.	
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

INTRODUCTION:DICAMBA

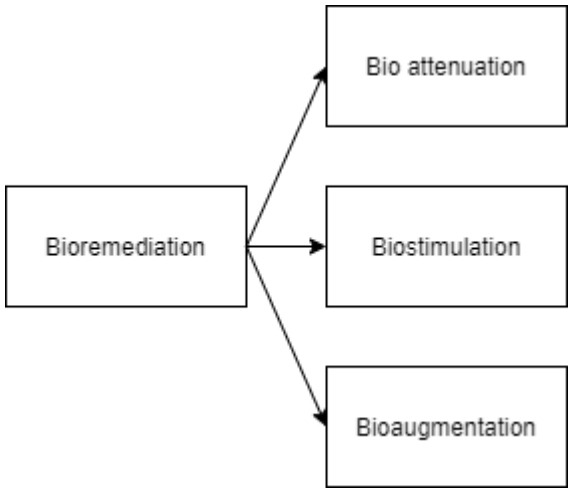
- **DICAMBA** is a benzoic acid herbicide that is used to control annual and perennial broadleaf weeds in grain crops and grasslands.
- Dicamba is soluble in water, and as such will leach into runoff water.
- Around 2016, Dicamba's use came under significant scrutiny due to its tendency to spread from treated fields into neighboring fields, causing damage.
- A crop that is sensitive to Dicamba: **Soybean**

Dicamba application on field

Dicamba drift damage on soybean leaves

INTRODUCTION:BIOREMEDIATION

- **Bioremediation**- process that utilizes microorganisms or their products in order to treat polluted sites.
- Purpose- restore them to their original condition



Depends on the natural processes of degradation

Based on the addition of nutrients in order to stimulate indigenous microbial populations

Based on the inoculation of given environments with microorganisms that possess certain capabilities –ex. degrade pesticides

Microbial degradation, the solution ?

Common pesticide degraders and the pesticides degraded	
Pseudomonas sp	Aldrin, Chlorpyrifos, DDT, Endosulfan, Parathion
Bacillus sp	Chlorpyrifos, DDT, Glyphosate, Methyl Parathion, Parathion
Flavobacterium sp	Diazinon, Glyphosate, Methyl Parathion, Parathion

INTRODUCTION

What about fungal pathogens ?

It is estimated that about 25% of global cereal production may be contaminated with mycotoxins

- *Fusarium* is a genus of filamentous fungi that can be found in plants and soils. The genus contains both saprophytic and pathogenic species (pathogens for wheat, barley, oat).
- *Fusarium culmorum* is a ubiquitous soil-borne fungus with a highly competitive pathogenic ability that can cause both Fusarium Head Blight (FHB) and Fusarium Seedling Blight (FSB).

Symptoms of FHB; blighting of the entire head

Symptoms of FSB; brown discoloration of roots and coleoptiles

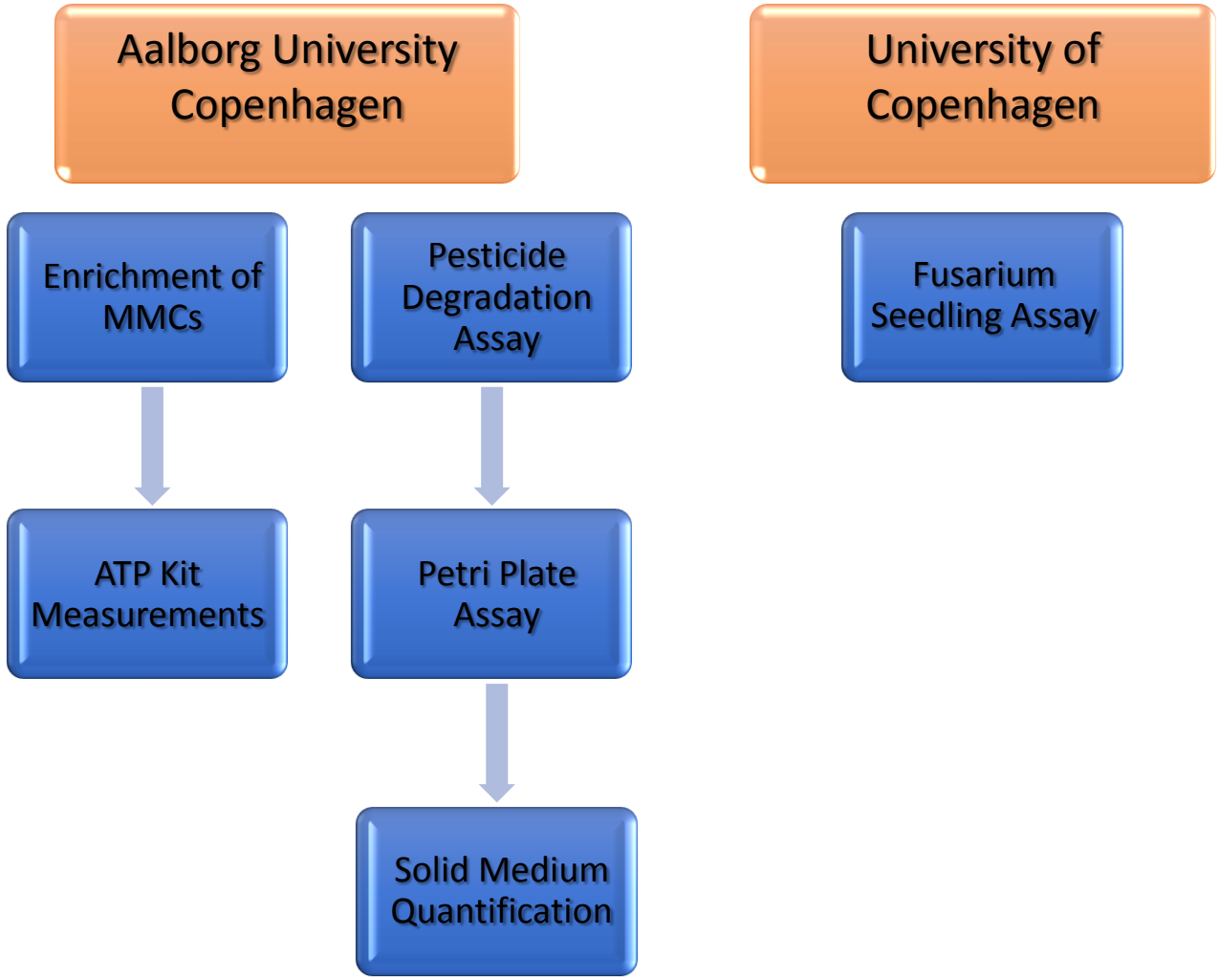
SCIENTIFIC QUESTION AND APPROACH

- Pesticides are compounds used to control different pests , but at the same time present several risks to sensitive crops, humans and the environment, since they can accumulate in soil, organisms or leech into groundwater.
- **Chr. Hansen**- working with *Bacillus* bacteria to provide greater yields in agriculture and protection against various pests; mostly use directed evolution to avoid GMO products
- **QUARTZO®**, **PRESENCE®** , **INTENSE®**
- A trend to develop plant protection products starting from microorganisms
- What if we could find ways to degrade the existing, soil accumulated pesticides while at the same time provide other pest control alternatives ?

SCIENTIFIC QUESTION AND APPROACH

- **Could we find bacteria that degrade the pesticide Dicamba, while at the same time have biofungicide abilities against *Fusarium culmorum* ?**
- To achieve that, the two functions are tested separately: pesticide degradation and crop protection
- Pesticide degradation: tested in liquid and on solid medium
- Crop protection: tested with wheat seedlings placed in sand pots, and inoculated with *Fusarium culmorum*
- Organisms that prove abilities to exert the dual function tested in a coupled experiment

EXPERIMENTAL SETUP



EXPERIMENTAL SETUP

- **Pesticide Degradation Assay**: Degradation efficiency

of the previously isolated strains (approx. 60) is assessed through a 2-week experiment. After preactivation in LB medium, the organisms are incubated at 30 degrees Celsius in M9 medium supplemented with 50 mg/L Dicamba as sole carbon source. Determination of the degraded amount is performed through HPLC analysis.

- **Enrichment** of mixed cultures from pesticide contaminated soil samples. 4 MMCs have been isolated from soil samples . Five grams of soil were added to 20 mL of Enhanced M9 medium and the cultures were passed every 7 days in order to wash out the soil, which left the pesticide as carbon source.

EXPERIMENTAL SETUP

- **Petri Plate Assay (Solid Medium Assay)** to increase the speed of screening and to preserve the cultures in minimal medium. Solid medium is Enhanced M9 Medium with 15 g/L Agar and Dicamba.
- **ATP Kit** measurements – BacTiterGlo™ Kit Promega to determine metabolic activity of the cultures.

EXPERIMENTAL SETUP

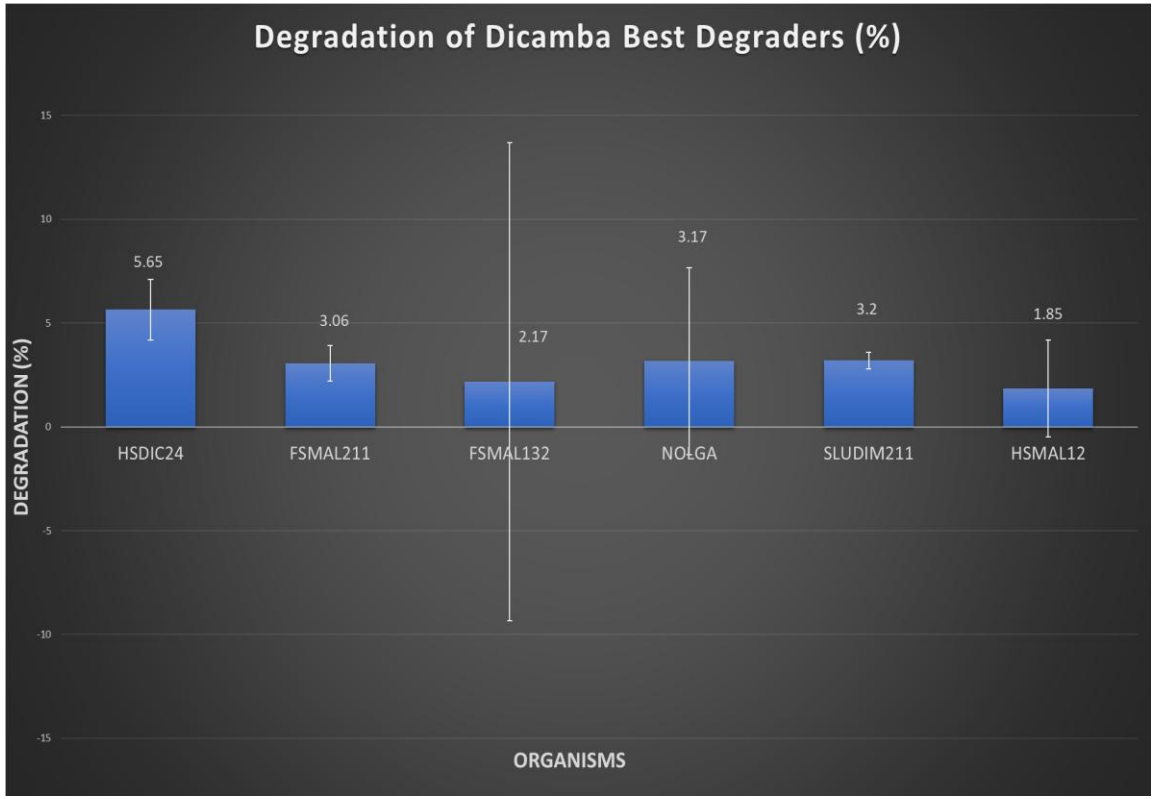
- **Fusarium Seedling Assay:** Wheat seeds coated with *F. culmorum* and biocontrol agents in sand pots- **Disease Index:** a unit determined by scoring the seedlings based on the severity of their symptoms.
- **Seedling Assay** with Dicamba application.

RESULTS

PESTICIDE DEGRADATION ASSAY

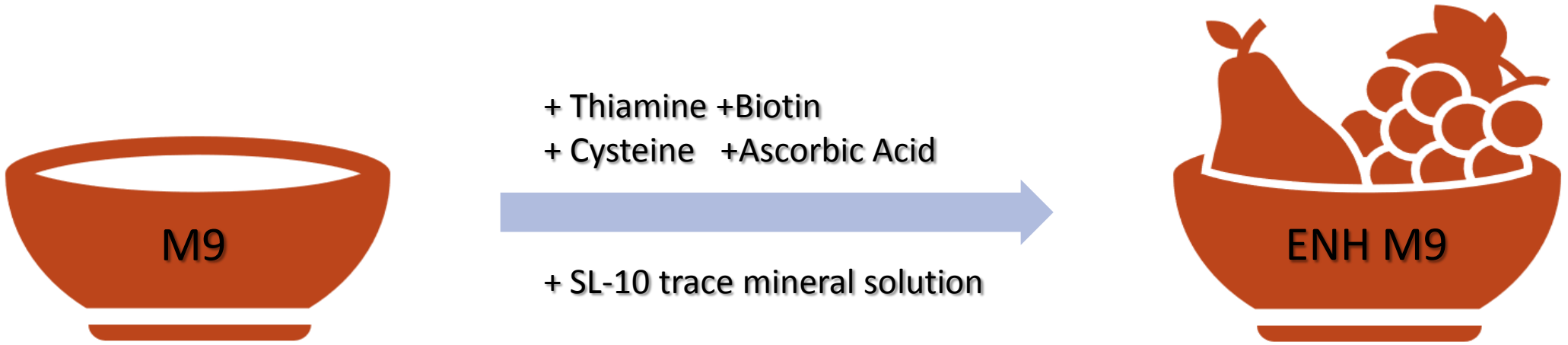
- Bacteria were tested in batches of about 10 at a time, in order to keep conditions homogenous
- Improvement in the method → Decrease in the error
- Very small negative values, mainly due to errors in calculating evaporation

RESULT OF THE EXPERIMENT: 6 POSSIBLE DEGRADERS



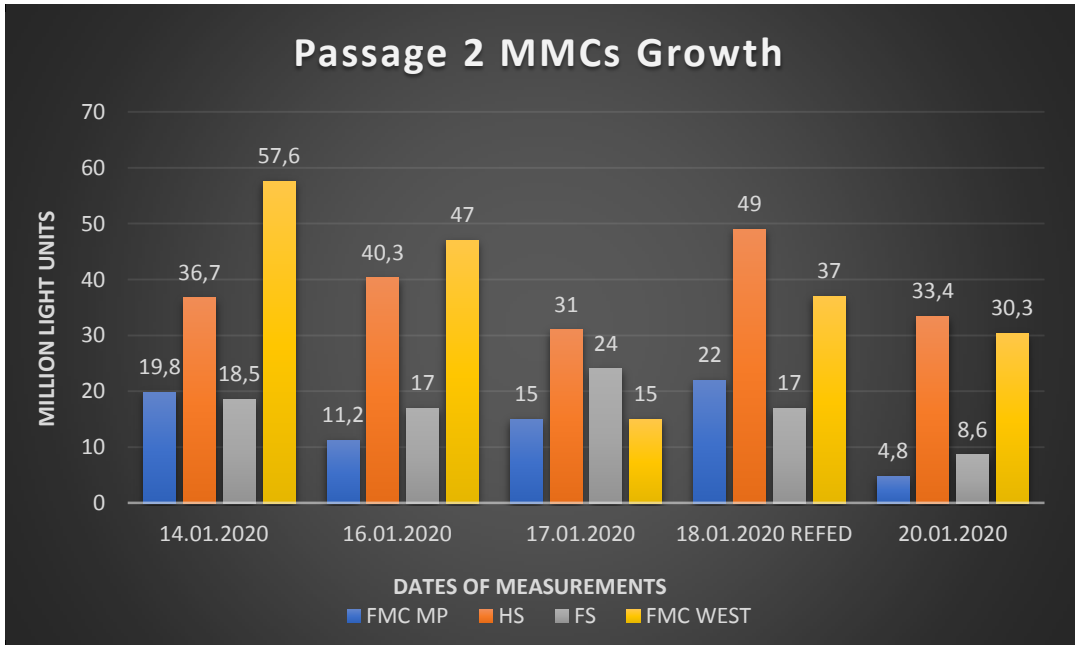
MEDIUM OPTIMIZATION

- The minimal medium mainly utilized in this study is M9 minimal medium with 50 mg/L Dicamba as sole carbon source.
- Some organisms may not properly grow on it
- Improved medium: contains amino acids, vitamins and trace minerals: Thiamine, Biotin Cysteine, Ascorbic Acid + SL-10 trace element solution→ **ENHANCED M9 MEDIUM**



ENRICHMENT PERFORMED ON SOIL SAMPLES

- 4 MMCs have been isolated from soil samples
- ATP Kit measurements to determine activity of the cultures
- REFED step: addition of 5 mL fresh Enhanced M9 Medium



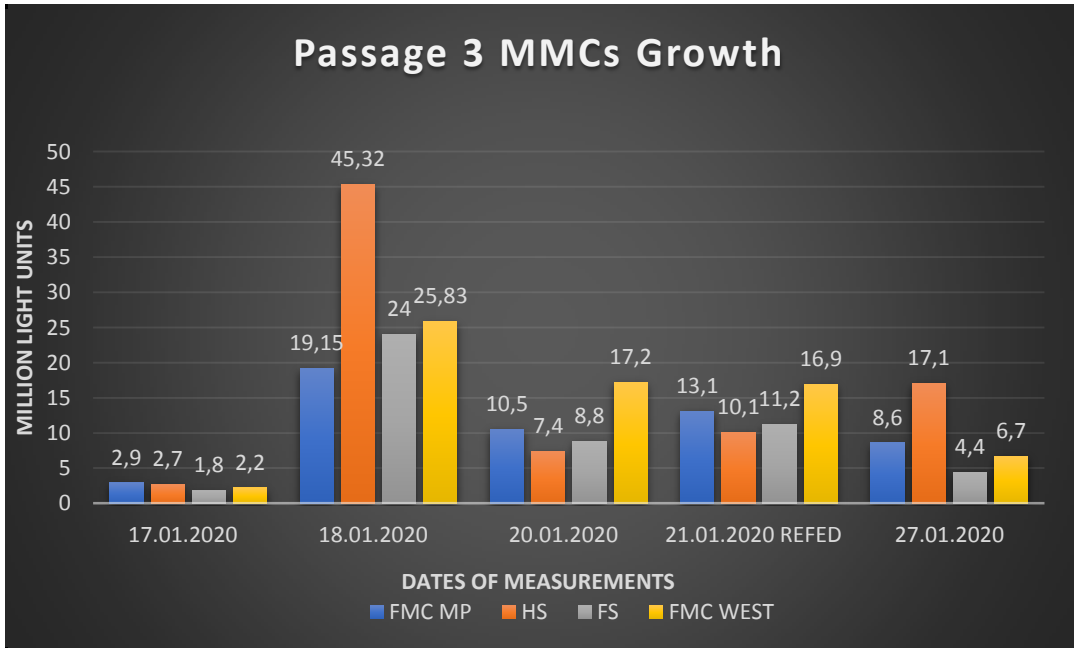
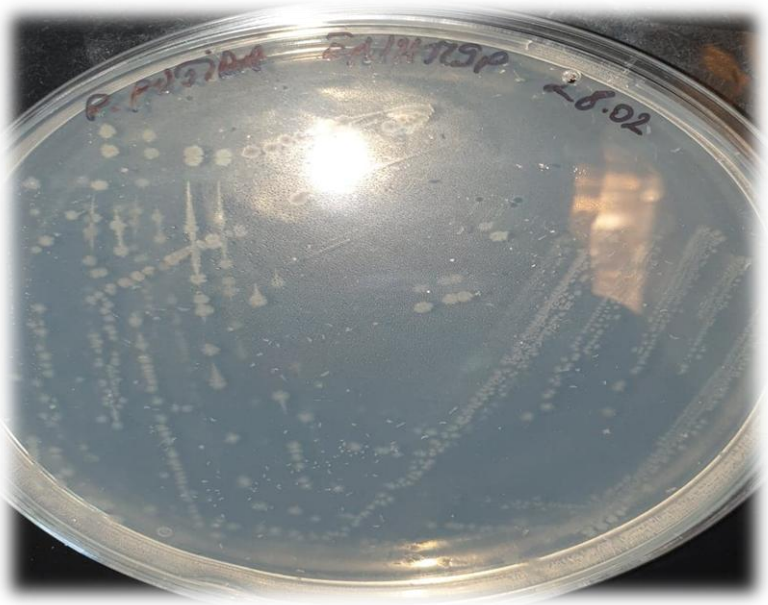


PLATE ASSAY AND BACTERIAL GROWTH

- Solid medium is Enhanced M9 medium with 50 mg/L Dicamba and 15 g/L agar
- Organisms preactivated in LB, washed, then plated and growth was assessed after 7 days

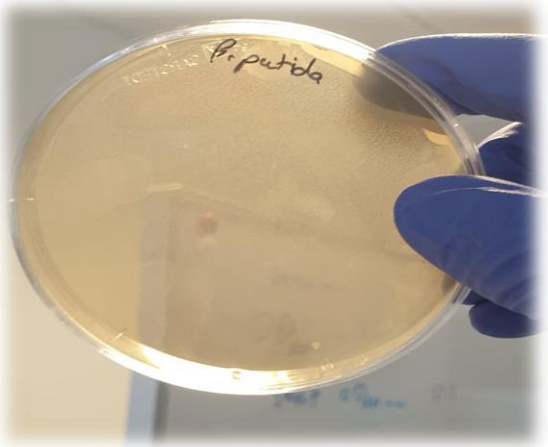
Pseudomonas putida



Micrococcus luteus

PLATE ASSAY AND BACTERIAL GROWTH

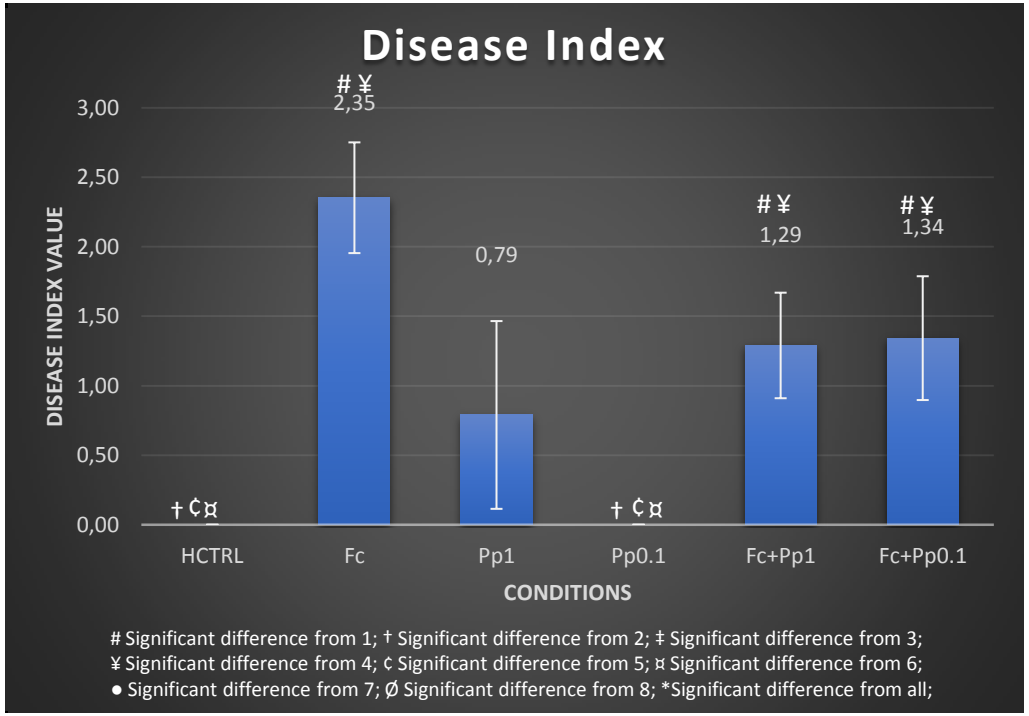
- A medium was devised in order to test if the cultures plated are utilizing the Dicamba or they are feeding on another compound (ex. Agar)
- The same Enhanced M9 Agar was used, but without the Dicamba.
- RESULT: **ORGANISMS MOST LIKELY GROWING ON THE PESTICIDE – SELECTIVE MEDIUM FOR PESTICIDE DEGRADERS**



FUSARIUM SEEDLING ASSAY EXPERIMENT I

DISEASE INDEX- calculated from the number of healthy/diseased seeds

Pseudomonas putida KT2440 was used as biocontrol agent
The seeds were coated with a suspension of the bacterium at either an OD of 1 or 0.1
The decrease in disease symptoms was not statistically significant (p= 0.10 and 0.14).



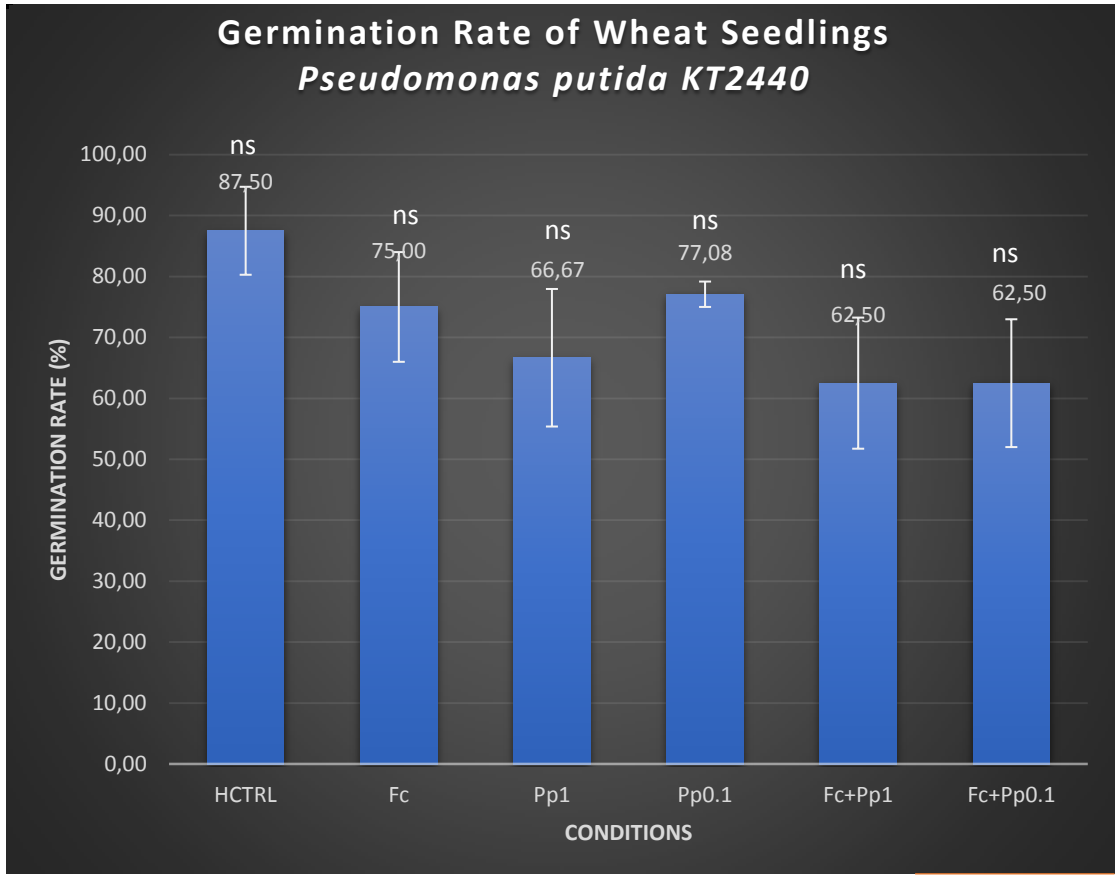
Healthy Control

Fusarium culmorum

Fusarium and *P. putida*

GERMINATION RATE

Pseudomonas putida KT2440 did not affect the germination rate of the wheat seedlings



FUSARIUM SEEDLING ASSAY EXPERIMENT II

- Three different organisms were used as biocontrol agents: *Bacillus subtilis, Micrococcus luteus* and a different strain of *Pseudomonas putida*
- The seeds were coated with a suspension of the bacterium at an OD of 1

HCTRL

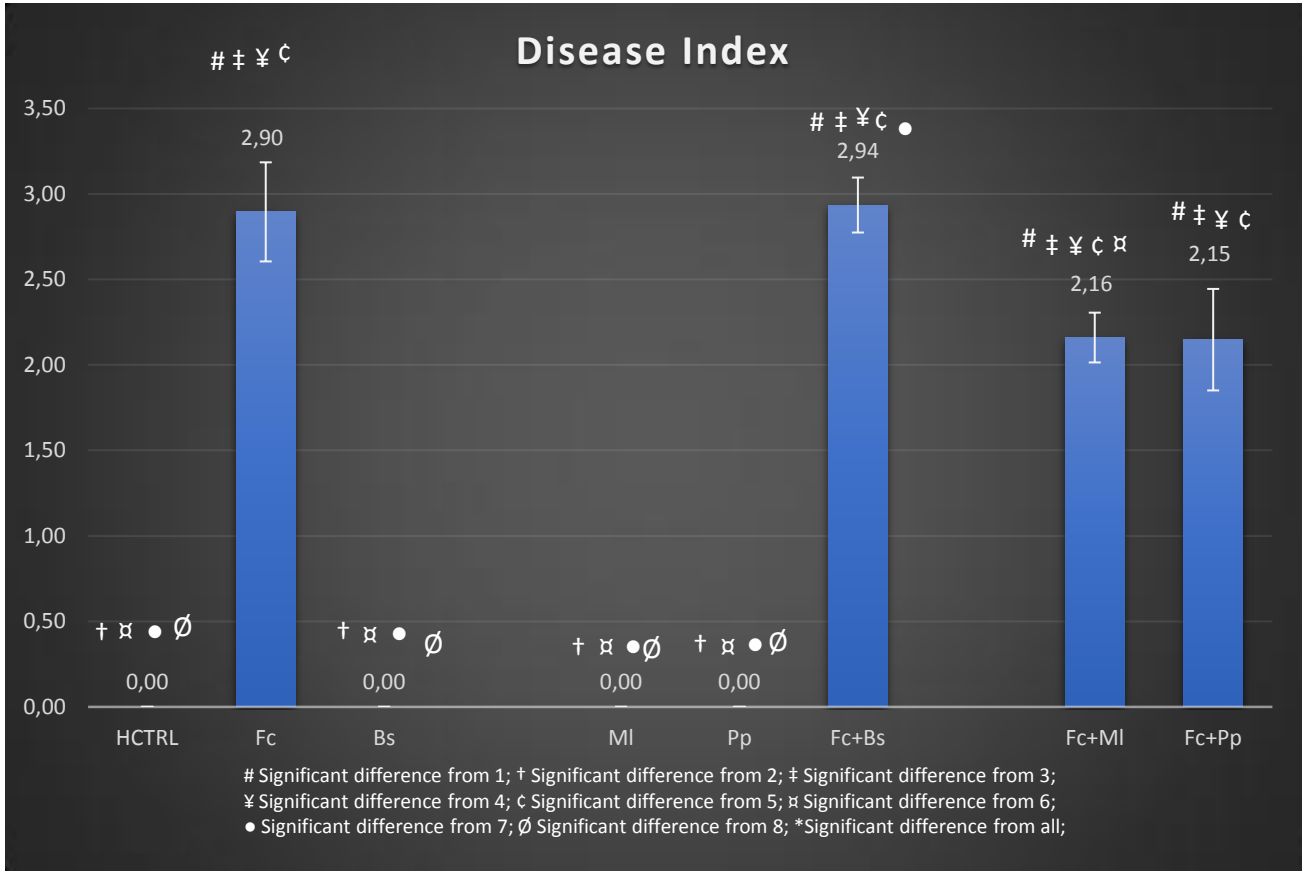
Fusarium culmorum

Fc+Bacillus subtilis

Fc+Micrococcus luteus

Fc+Pseudomonas putida

FUSARIUM SEEDLING ASSAY ROUND II



Three different organisms were used as biocontrol agents

Bacillus subtilis, Micrococcus luteus and a different strain of *Pseudomonas putida*

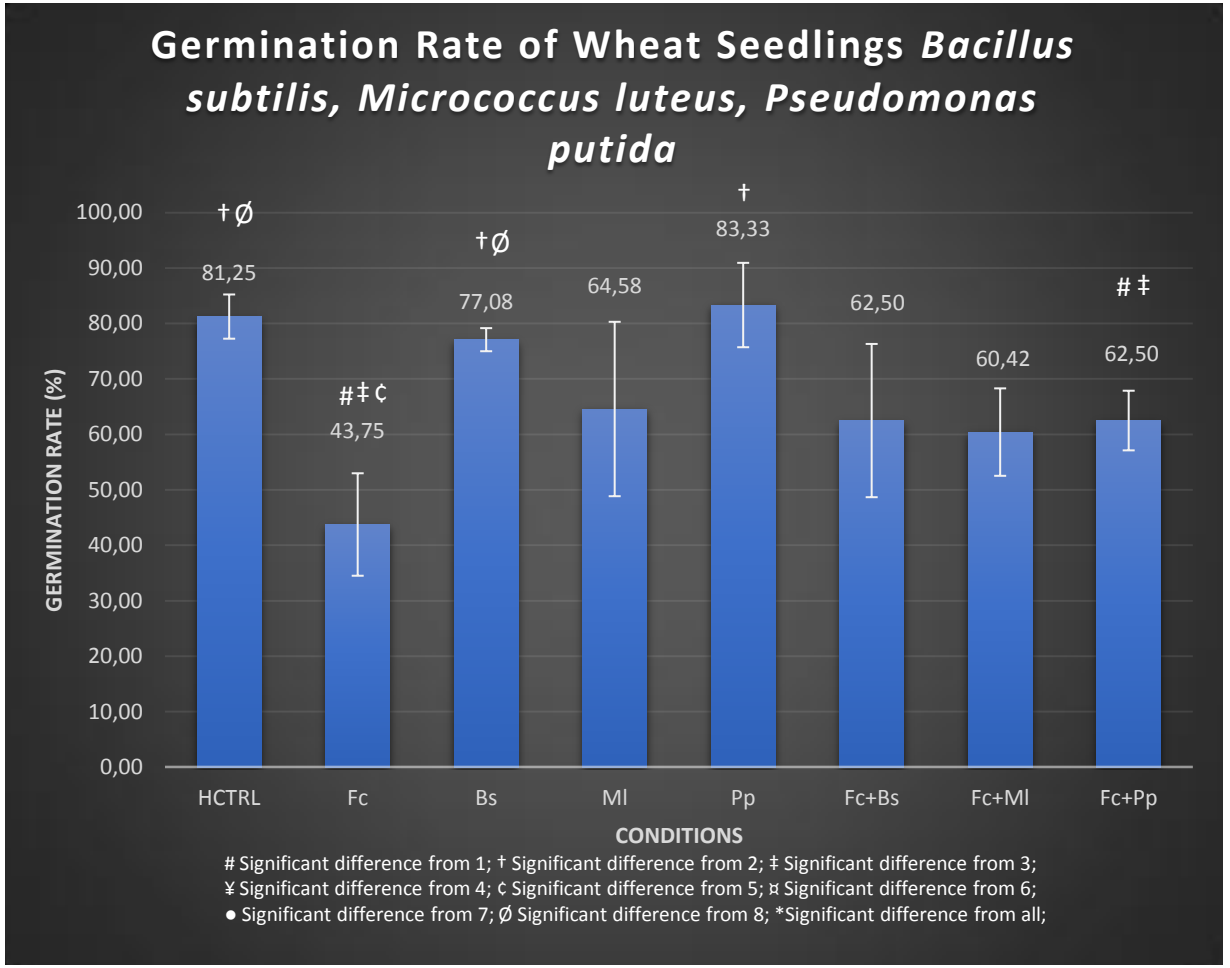
The decrease in disease symptoms was not statistically significant

For *Bacillus subtilis*, p= 0.90; For *Micrococcus luteus,* p= 0.06; For *Pseudomonas putida,* p= 0.12;

GERMINATION RATE

The *Fusarium culmorum* only condition exhibited a statistically significant decrease in germination rate compared to the control sample (p= 0.009)

The rest of the conditions show no significant decrease in germination rate with the application of the biological control agents



DICAMBA APPLICATION

Application of Dicamba at different concentrations to determine if any of the concentrations are suitable for future work

CTRL

Suitable concentration: **6.25 mg/L**

50 mg/L

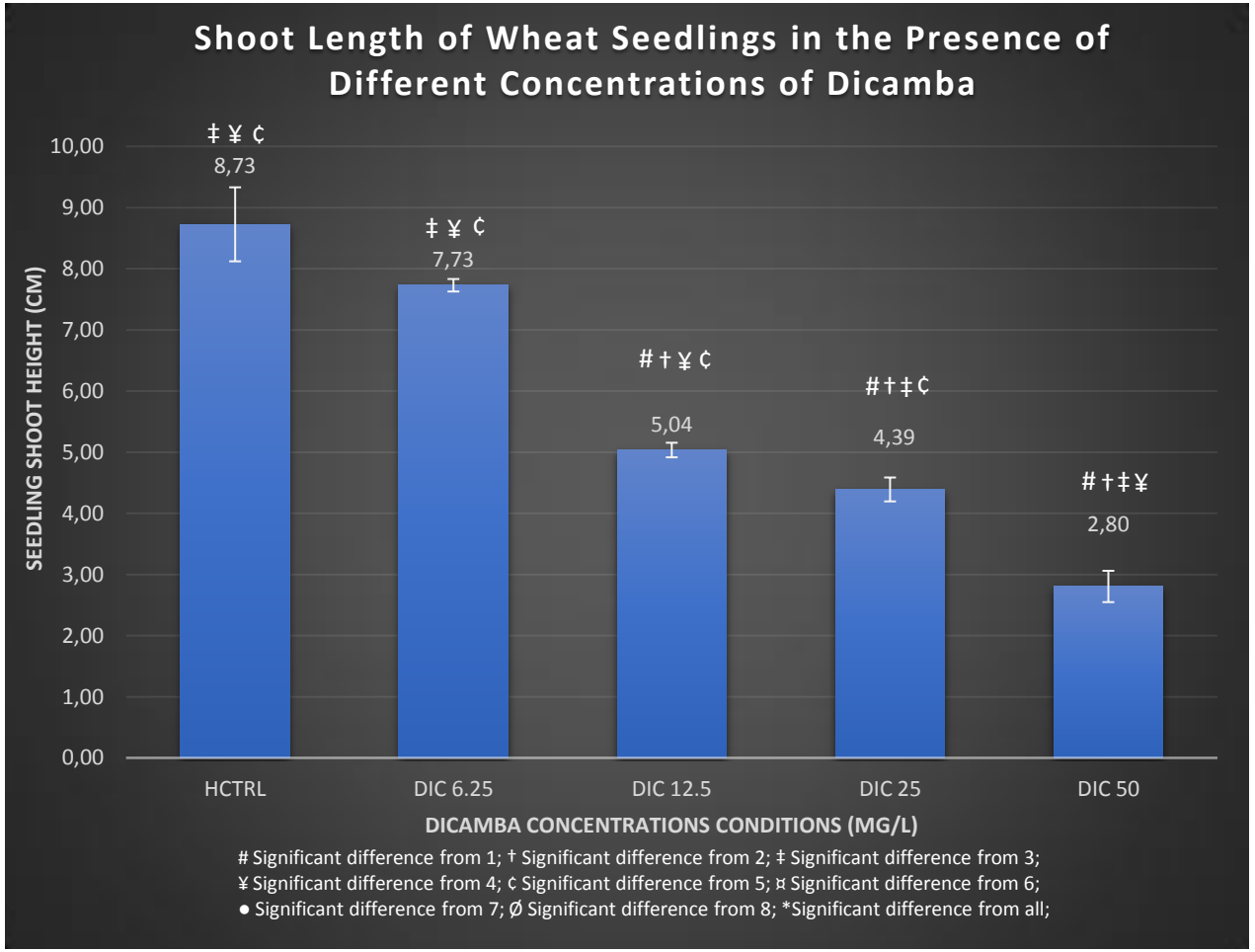
25 mg/L

12.5 mg/L

6.25 mg/L

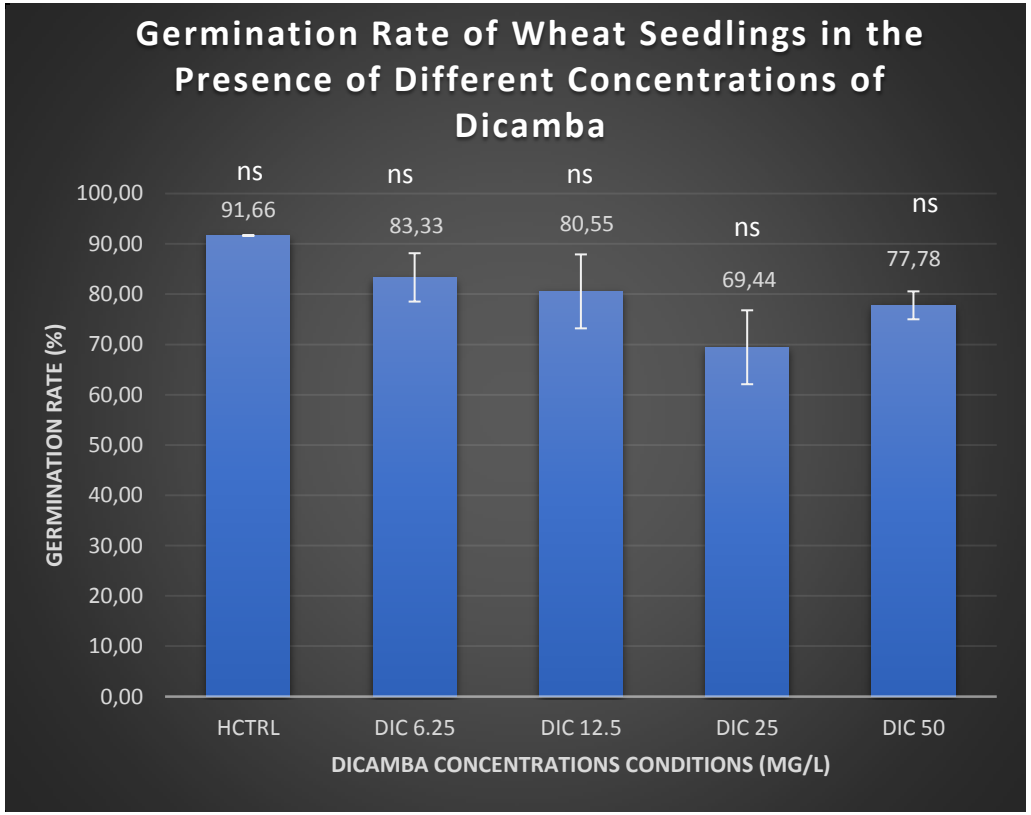
DICAMBA APPLICATION

- With the increase in Dicamba concentration (0 to 50 mg/L), there is a significant decrease in shoot length, aside from the 6.25 mg/L concentration.
- 6.25 mg/L is the only viable Dicamba concentration, since it would not greatly interfere with the assessment of the Fusarium Seedling Blight symptoms.



DICAMBA APPLICATION:GERMINATION RATE

Even though the effect of Dicamba on shoot and root length and aspect was severe, it did not interfere with the germination rate.



FUTURE PERSPECTIVES AND CONCLUSION

FUTURE PERSPECTIVES

- **Pesticide Degradation Assay** → IMPROVED
  - New Enhanced M9 medium used instead of regular M9 medium
  - Increased volume of conditions from 20 mL to 50 mL
  - Increase duration of the experiment from 2 weeks to 1-2 months
- **MMCs** → Tested through both the Pesticide Degradation Assay and the Seedling Assay to determine properties
- **Solid Medium Assay** → viability of the quantification method on solids to be determined: What is the recovery rate of Dicamba ?
- **Coupled experiment** → Seedling Assay with added Dicamba at a viable concentration
- Many more organisms were to be tested using the Seedling Assay, preferably all pesticide degraders found

CONCLUSION

- Main goal was to find organisms that can degrade Dicamba and at the same time provide biological control against *Fusarium culmorum* → **DUAL FUNCTION**
- Dicamba degradation: *Bacillus subtilis, Pseudomonas putida, Micrococcus luteus* and FSMAL132 were able to degrade Dicamba → Solid Medium Assay: Unable to quantify at this point

- Biological control: *Pseudomonas putida KT2440* and *Micrococcus luteus* seemed to decrease the symptoms of Fusarium Seedling Blight, but the results were not statistically significant
- At this point unable to determine dual function → screening will continue

ACKNOWLEDGEMENTS

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THANK YOU ！