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Biotic priming of growth and stress tolerance in cereals

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ABSTRACT

Instead of using chemical pesticides for plant protection there is the possibility to activate plants' innate immunity defense against pests and diseases using bacteria. Many bacteria have been tested for their priming potential while not much research has assessed the priming effect against abiotic stress factors. In this investigation four bacterial strains of *Bacillus amyloliquefaciens* namely FZB42, 5113, 5033 and 5036 were tested and evaluated under cold stress (-4°C) conditions. The strains 5033, FZB42 and 5113 showed a statistically significant difference in chlorophyll content after cold stress treatment. The possibility of increased targeting with the nano material titanium dioxide (TiO_2) was also tested. TiO_2 treatment increased bacterial spore germination on LB-agar plates.

POPULAR SCIENTIFIC DESCRIPTION

In this study bacteria, living in symbiosis with wild plants, were transferred to agricultural plants to see if they have a positive effect on stress factors from the environment or any other possible factor. Freeze stress was the main factor investigated and some of the results show a significant defense inferred by the bacteria. Also a pilot study investigating the possibility to encapsulate the bacteria with a nano-material to increase its ability to stick to the plant and to survive was conducted. The results of the encapsulation were inspiring but there were no statistically significant differences so the effects are yet to be proven.

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1.0 INTRODUCTION

Toxic substances that are released into nature by man have been listed as one of the 9 most important threats to the planet and humanity (Rockström 2009). In agriculture substantial quantities of toxic compounds are used to counteract pests and weeds, these agrochemicals constitute health hazards if present in food and may easily leak out to groundwater recipients and finally into the oceans. Hence it is of utmost importance to reduce the use of chemical substances in agriculture. According to FAOstat the sum of the use of all common pesticides, in the 52 questioned countries, corresponds to 272 941 tons. Bacterial priming of plants is a way of substituting the chemicals by initiating processes known as induced systemic resistance (ISR) or systemic acquired resistance (SAR) in plants. Stimulation of ISR has been shown to be effective against attacks from fungi, bacteria, viruses, nematodes, parasitic plants and insects (Wang et al. 2009). Both ISR and SAR are latent defenses that exist in all plants but differ in signaling and the nature of the active compounds. SAR is dependent upon the salicylic acid signaling pathway and activates a set of genes in which the NPR1 gene is most prominent, while ISR is dependent upon the jasmonic acid signaling pathway. These two pathways, although different in the early signaling steps, converge at the NPR1 gene being a key regulator of SAR and ISR. Otherwise ISR does not directly activate any genes although it increases the level of a few pathogenesis related (PR) proteins, such as peroxidase, chitinase and β -1-3 glucanase, upon pathogen infection. Lignifications of the plant cell walls have also been found after priming (Yuen et al. 2009). Virulent pathogens have evolved ways to circumvent the plant defense systems either by suppressing resistance actions, withstanding resistance action or by not initiating the defenses at all (van Loon et al. 1998). It is believed that the most common evasion strategy is the latter; hence activating the latent defenses in plants is an appropriate strategy of controlling diseases in plants. Apart from having this priming effect, *Bacillus amyloliquefaciens* strains are also often known to serve as so called plant growth promoting bacteria (PGPB). The known mechanisms of plant growth promotion are bacterial synthesis of plant growth hormones, such as indole-3-acetic acid (IAA), cytokinin and gibberellins; reduction of the volatile plant hormone ethylene by production of 1-amino cyclopropane-1-carboxylate deaminase; and through an increased uptake of nutrient from the soil (Wang et al. 2009). Bacterial priming may also be beneficial against abiotic stresses but little information is available on this subject, even less for such effects on cereals. Chilling and freezing stress are common problems in Nordic agriculture. When temperatures drop close to 0°C (chilling) or below 0°C (freezing) plant tissues are under severe risk of damage. Lesions appear on the leaves because of damaged membranes and chloroplasts. The photosynthetic rate and the respiration rate are reduced. Protein synthesis is inhibited and proteins are degraded. The plants respond to cold by accumulating abscisic acid (ABA) which starts the onset of approximately 100 different genes. The most important mechanisms are production of glycoprotein and sugars to prevent ice crystallization and the substitution of fatty acids in the polypeptide membranes to increase elasticity.

In this investigation the effects of priming with *Bacillus* against freeze stress were assessed. *Bacillus* strains are known to prevent phytopathogens in three different ways: direct antagonism against pests and fungi; promotion of plant nutrition and growth; and induction of plant defense (Choudhary & Bhavdish 2008). In the soil there is an intricate network of communication between plant roots, beneficial bacteria, beneficial fungi, deleterious bacteria and deleterious fungi. Plants produce root exudates that are sensed by both bacteria and fungi and plants respond to compounds produced by microorganisms (see fig. 1).

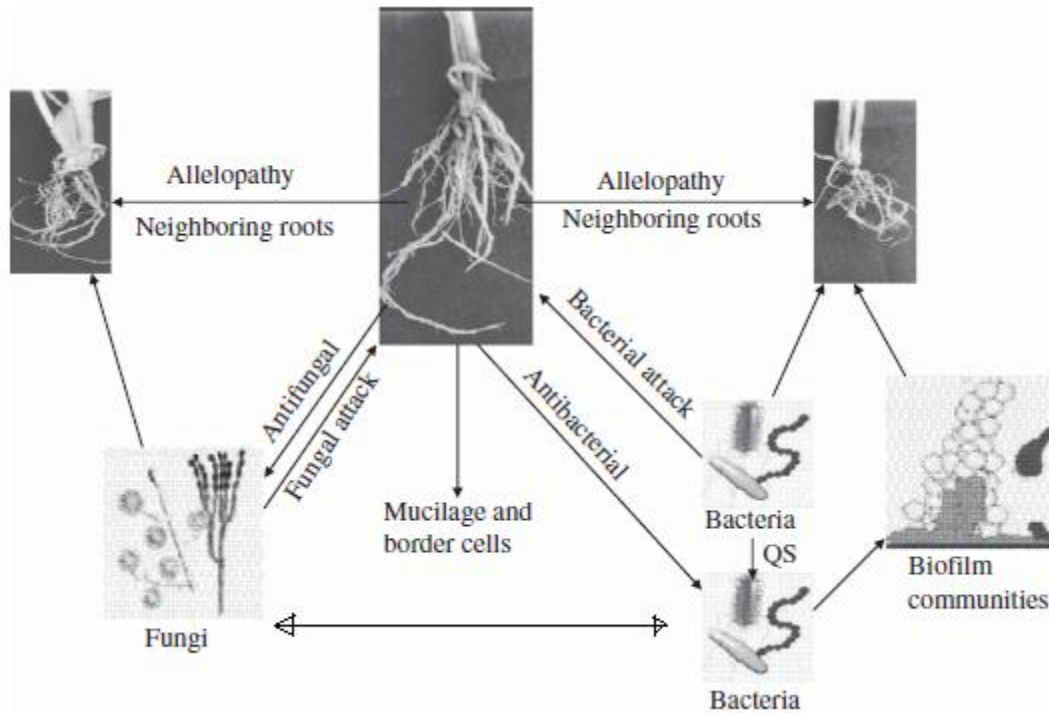


Fig. 1. Schematic picture of the intricate interplay between roots and other organisms in the rhizosphere. QS, quorum sensing. An additional arrow has been added to indicate interaction between fungi and bacteria. For example it is common for beneficial fungi and bacteria like *Trichoderma* and *Bacillus* to produce anti-fungal or anti-bacterial substances like polyphenols and glucanases, respectively. Source: Walker et al. (2003)

Nanotechnology has been developed during the last 20 years. In 2006, there were over 600 products using nanotechnology on the global market in a myriad of applications such as drugs, cosmetics and super materials. In spite of this only limited toxicological data are available, but to date no unique or distinctive toxicity of nanomaterials have been identified (UNEP year book 2009). However it has been found that titanium dioxide (TiO_2) exposed to UV-light can catalyze the conversion of water to hydrogen peroxide (H_2O_2) and hydroxyl radicals which breaks membranes and lyse cells (Minhua et al. 1998). These nano materials will probably end up in water recipients and there are reasons to be concerned about their toxicity. For example Hartmann et al. (2010) found growth inhibitory effects of TiO_2 on the green algae *Pseudokirchneriella subcapitata*. In their report they emphasized the importance of size of the compound for a toxic effect. Also Yeung et al (2009) reported anti-microbial properties of TiO_2 on *Bacillus subtilis* and *Escherichia coli*. Although, considering that these inhibitory effects require UV-radiation and that TiO_2 nano sol-gel might be a way to increase efficiency of priming, by increasing targeting and survival of bacterial spores applied to plants, and hence lowering dosage, nano material might be a formidable benefit. Similar things have been successfully conducted in medicine with increased drug targeting but never before has it been tested on the mutualistic relationship between plants and certain bacteria. Hence the effect of bacterial spore encapsulation with TiO_2 was tested on six cultivars of barley, oat and wheat.

2.0 AIMS

To test different *Bacillus amyloliquefaciens* strains on different cultivars of cereals to screen for highly beneficial interactions.

To test the effect of TiO₂ on the cereals and on the bacteria.

To study how the bacteria colonize the plants.

3.0 METHODS

3.1 BIOTIC PRIMING

Six cultivars were used, two of each of the species *Triticum aestivum* (Vinjett and Kranisch), *Hordeum vulgare* (Tipple and Mitja) and *Avena sativa* (Belinda and Kerstin). These cultivars have been developed in Sweden and adapted for the conditions in the northern agricultural parts of Sweden. Four strains of *Bacillus amyloliquefaciens* were tested namely FZB 42, UCMB-5113, UCMB-5033 and UCMB-5036. Strain FZB 42 is a fully sequenced and commercially available PGPB. The other strains were isolated by Reva et al. (2004). Strain 5033 and 5036 were both isolated from cotton plants from Tajikistan and 5113 was isolated from soil of the Karpaty Mountains. Suitable stress conditions were established in growth chambers before experiments with priming were initiated. Bacteria were cultivated primarily as a mini culture in LB-media for approximately 20 hours. The mini culture was transferred into a larger volume of LB-media and cultivated for another approximate 20 hours. To induce spore formation the cultures were exposed to 80⁰ C for 10 minutes. Finally the cultures were centrifuged at 12000 rpm for 10 minutes and the pellet was resuspended in sterilized Milli-Q water. Seeds were obtained from Lantmännen AB in Uppsala and came from last year harvest. As control untreated seeds and the commercially available seed treatment were used. The treated seeds were either treated with chemicals or with a bacterial formulation with *Pseudomonas* bacteria (Cedomon). The seeds were dipped and incubated on a shaker for 2 hours at 28⁰ C in 10⁷ CFU/ml with the strains 5113 and 5036 or 10⁶ CFU/ml of the strains FZB42 and 5033. In the repetition the incubation time was extended to 4 hours because of a mechanical failure of the shaker. The seeds were then put on filter paper to germinate. This was to guarantee enough replicates without using valuable space in the growth chamber and also to document variation in germination between treatments. From each Petri dish 10 of the most developed seeds were picked and put into pots with 5 seeds in each pot.

3.2 GROWTH CONDITIONS

All plants were grown in growth chambers with 22⁰ C and 15 hours of light at 200 $\mu\text{mole m}^{-2}\text{s}^{-1}$. The soil used was a dry sterilized soil with one third of vermiculite. Five seeds were planted in 8x8x5 cm pots. For stress treatments the plants were moved into Percival growth cabinets with stable conditions and the possibility to log temperature, light and air humidity. After the trials, with 5, 8, 12 and 24 hours in -4⁰ C, 12 hours was found to be the most suitable stress treatment. A strong edge effect was apparent but that was successfully evaded by wrapping the plants and trays with plastic on all sides.

3.3 SPAD MEASUREMENT

After stress treatment a SPAD-meter was used to measure differences in chlorophyll content in the leaves. The SPAD-meter is a spectrometer calibrated to measure chlorophyll and gives an index from 0.0-99.9. A fully fertilized and healthy cereal has an approximate SPAD-value of 50. All leaves that survived were measured at the tip and the base of the leaf, an average was made and noted directly for the whole plant.

3.4 COLONIZATION

To investigate how the bacteria spread and colonize plants inoculated with 5113, two cross sections were made in vicinity to each node along the stalk. The cross sections were examined under a TM-10000 scanning electron microscope. Leaves from each node were also examined. To protect the plant material from deterioration sputtering with gold was used.

3.5 NANO ENCAPSULATION

Strain 5133 as a spore solution was mixed with TiO₂ and seeds and incubated for 1 hour at 28⁰ C on a shaker. The nano material was a TiO₂ sol-gel prepared by Gulaime Seisenbaeva at the Chemistry department SLU. The liquid contains 20% ethanol and is made to be diluted 1:9 with salt solution. MgCl₂ with 100mM concentration was used to dilute to stimulate natural biological conditions. The plants were grown in growth chambers and were compared and evaluated after five weeks and ten weeks. A CFU-experiment was also conducted on LB-agar plates to study the effect of TiO₂ on bacterial germination. The spore solution was mixed 50:50 with water as control named **d**; 50:45:5 with water and 2% ethanol named **c**; 50:45:5 with water and TiO₂ named **b**; 50:45:5 100mM MgCl₂ and TiO₂ **a**. Colonies were counted after two days and the experiment was repeated three times.

3.6 STATISTICAL ANALYSIS

All statistical analyzes were carried out with Minitab solutions 15. A one way ANOVA was used for analysis of the priming effect on chlorophyll content.

4.0 RESULTS

4.1 SEED GERMINATION

Seeds with bacterial treatments showed retarded germination compared to control except for in a few cases. After two days all cultivars, except Kerstin, treated with 5113 had started to germinate at a higher degree than all the other strains. Kerstin had very poor germination in all treatments including the control. After three days Belinda primed with both 5033 and 5113 were better than control and 5113 seemed to promote shoot growth. Mitja showed no greater differences between treatments. All treatments, except the chemical treatment, were similar to control although one could see a few more shoots in the 5033 treatment. Tipple germination was favored by all four strains and their seedlings had more shoots than control. Kranisch had fast and vigorous germination and 5113 was similar to control. Both wheat cultivars had a good interaction with FZB42 and Vinjett-FZB42 had similar germination as control and 5113 (See fig. 2 and appendix 1).

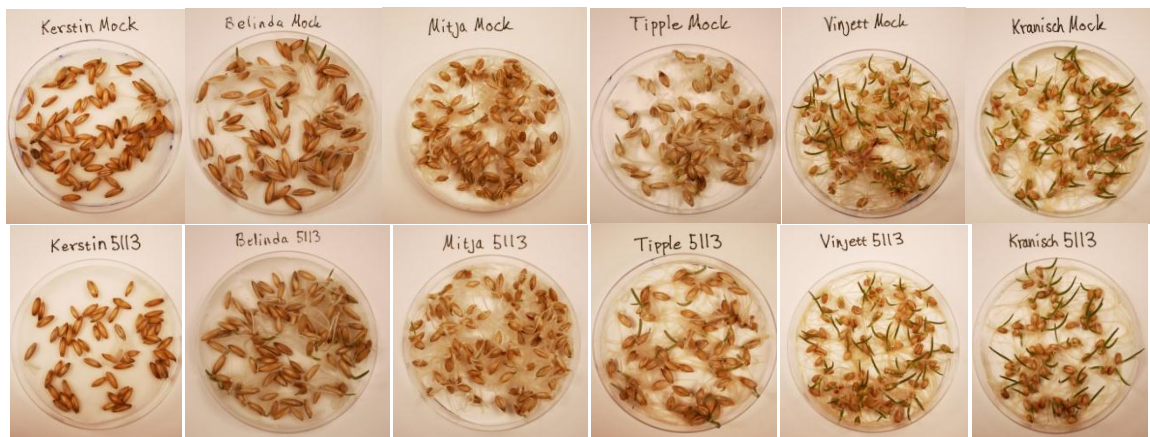


Fig 2. A comparison of all cultivars. Appearance of control (mock treated seeds) compared to 5113 treated seeds after 3 days of germination. Control plants in the top row and 5113 treated in the bottom row. From left to right: Kerstin; Belinda; Mitja; Tipple; Vinjett and Kranisch.

4.2 SPAD-INDEX OF FREEZE STRESSED PLANTS

After isolating the trays with plastic all plants seemed equally stressed to the naked eye but just in case the placement of the plants had been randomized. As indicated by the germination, wheat and FZB42 had a positive interaction and the wheat plants came out of the stress conditions with high chlorophyll content. There was a statistically significant difference between FZB 42 treatment and the chemical Celest treatment which had the lowest chlorophyll content (see fig. 3).

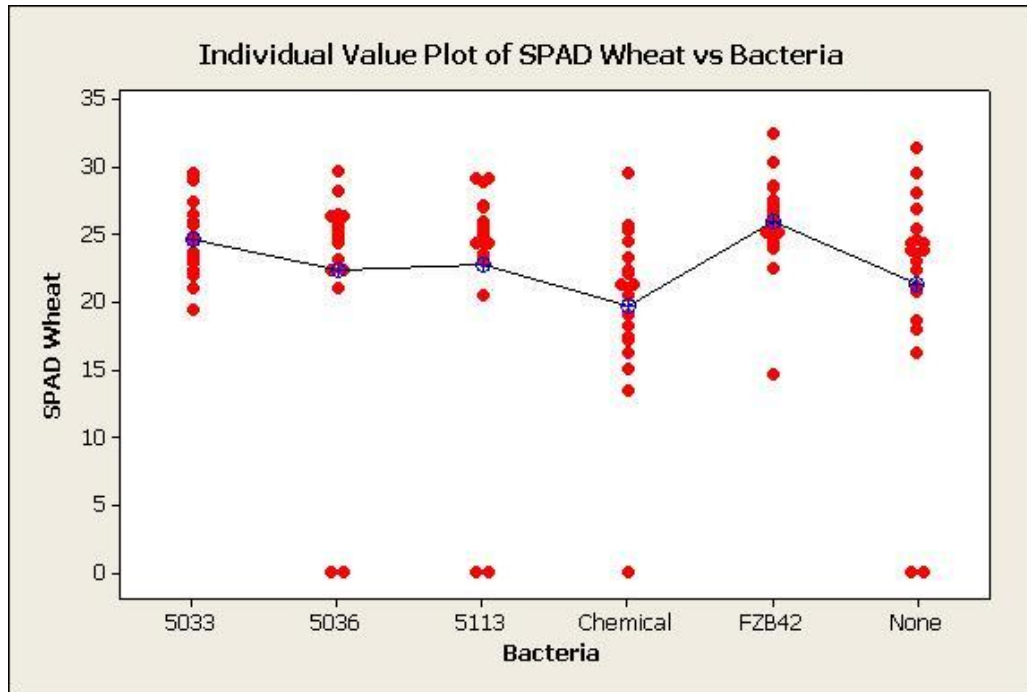


Fig 3. Individual value plot with means of SPAD-index for wheat. Zero indicates plants that died during cold stress. $P=0,044$.

For barley the opposite results were obtained and FZB42 treated plants had the lowest chlorophyll content (see fig. 4). There was a significant difference between FZB42 and 5033 which had the highest chlorophyll content. Also the 5113 treatment had a statistically significant difference to the FZB42 treatment and might also be considered the best for barley since it had a higher planted germination rate: 20/20 compared to 18/20 for 5033 (not a statistically significant difference, results not shown).

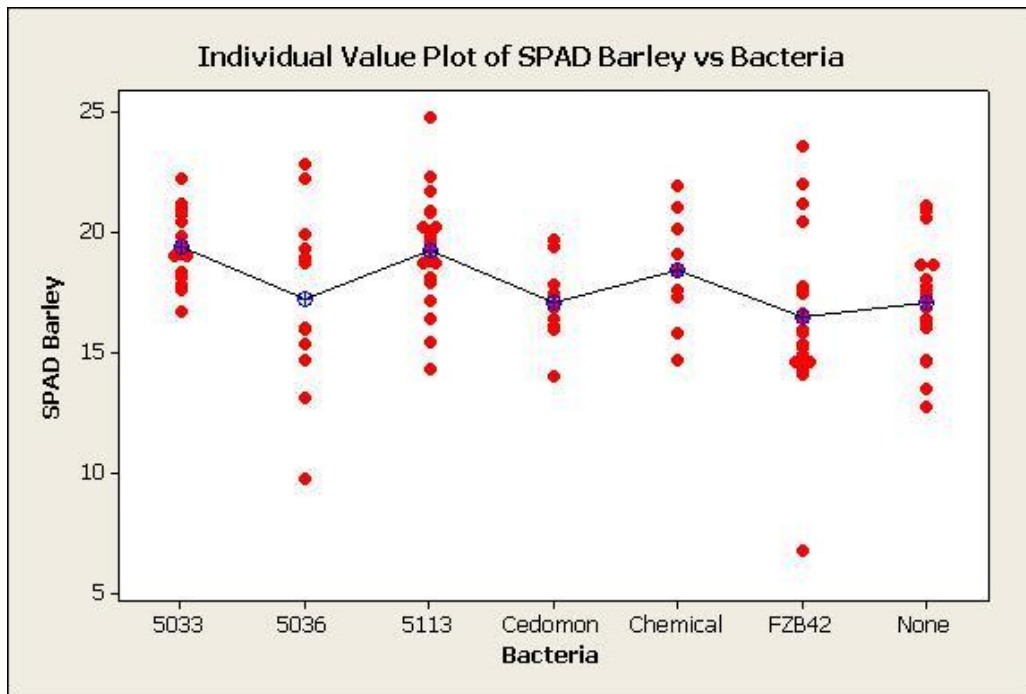


Fig 4. Individual value plot with means of SPAD-index for barley. $P=0,003$

Experiments with oat did not show any statistically significant differences between treatments. The 5033 treatment had the highest mean but none of the Kerstin seeds treated with 5033 that were planted actually grew. All values included in the statistics for 5033 were hence from Belinda plants (see fig. 5).

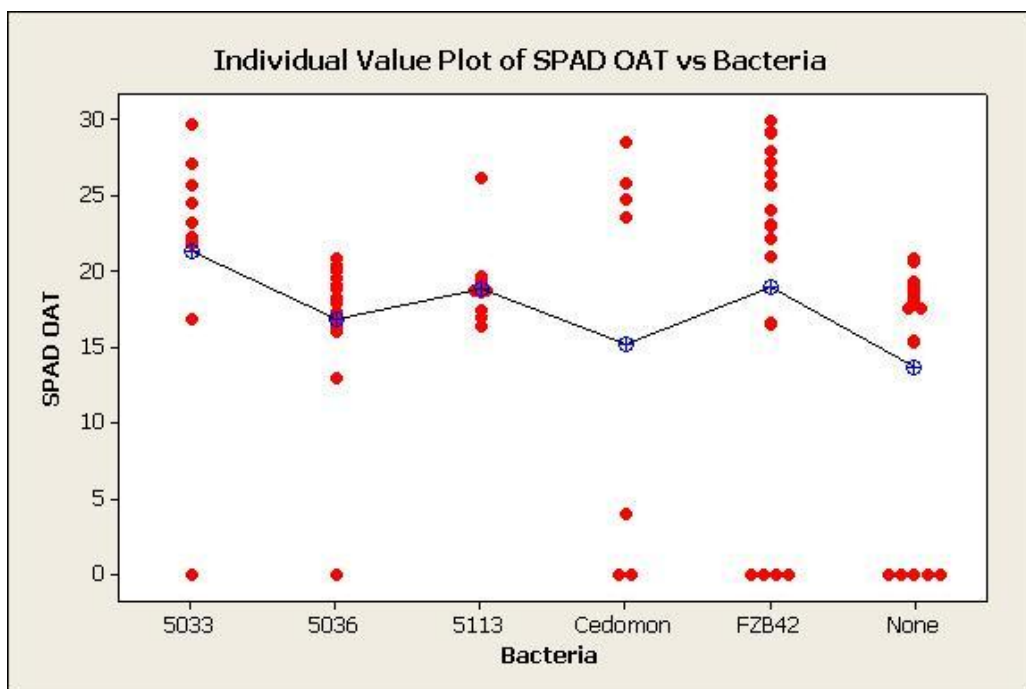


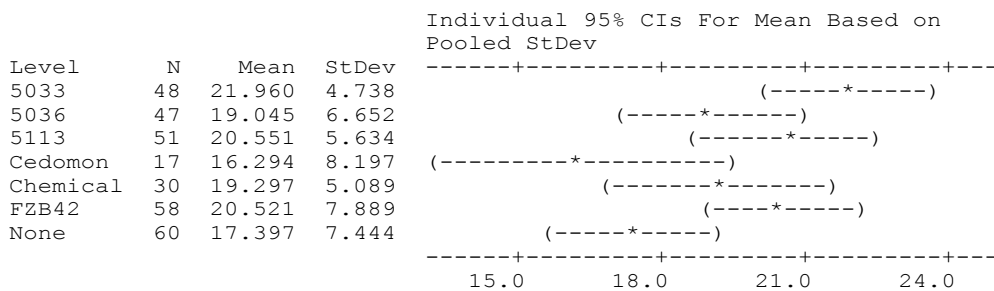
Fig 5. Individual value plot with means of SPAD-index for oat. Zero indicates plants that died during cold stress.

When all treatments and cultivars were included in the analysis, 5033 had the highest mean, lowest standard deviation and was the only treatment with a statistically significant difference to the control (see fig. 6 and table 1).

Table 1. Sstatistical analysis of all treatments and cultivars together.

Source	DF	SS	MS	F	P
Bacteria	6	856.8	142.8	3.26	0.004
Error	304	13320.2	43.8		
Total	310	14177.0			

S = 6.619 R-Sq = 6.04% R-Sq(adj) = 4.19%



Pooled StDev = 6.619

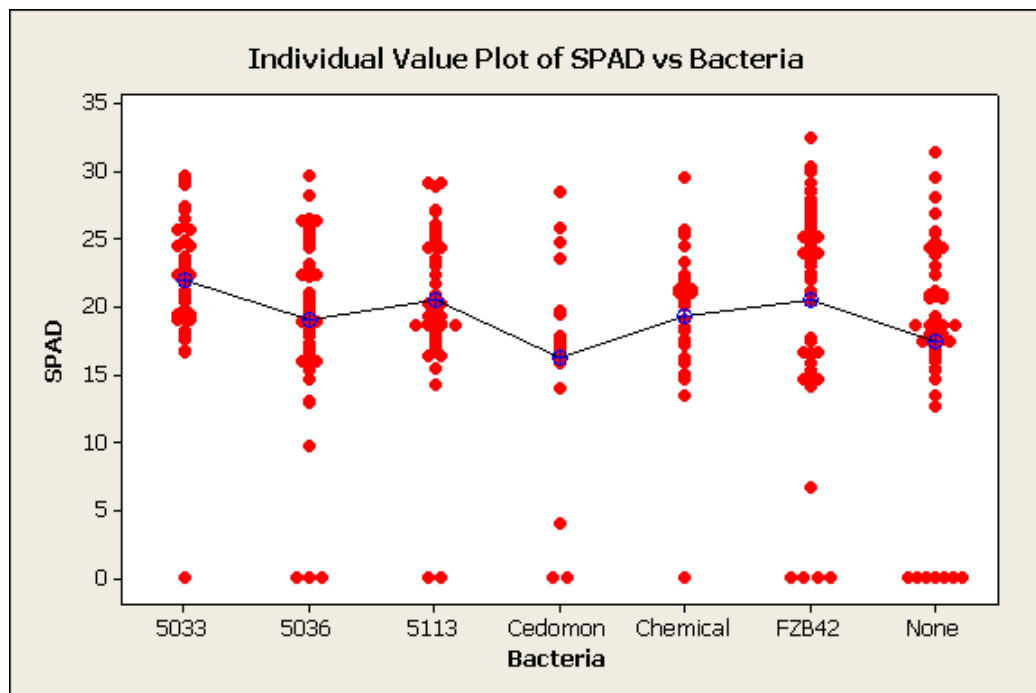


Fig 6. Individual value plot of SPAD-index for all treatments and cultivars.

4.3 SURVIVED LEAVES AFTER FREEZE STRESS

During the freeze stress many leaves died while some leaves got lesions but survived and regained or came through undamaged. There was some variation in the number of leaves that were still thriving after cold treatment. Strain 5113 and control showed a higher leaf survival than the other treatments. No statistically significant differences were apparent (fig. 7).

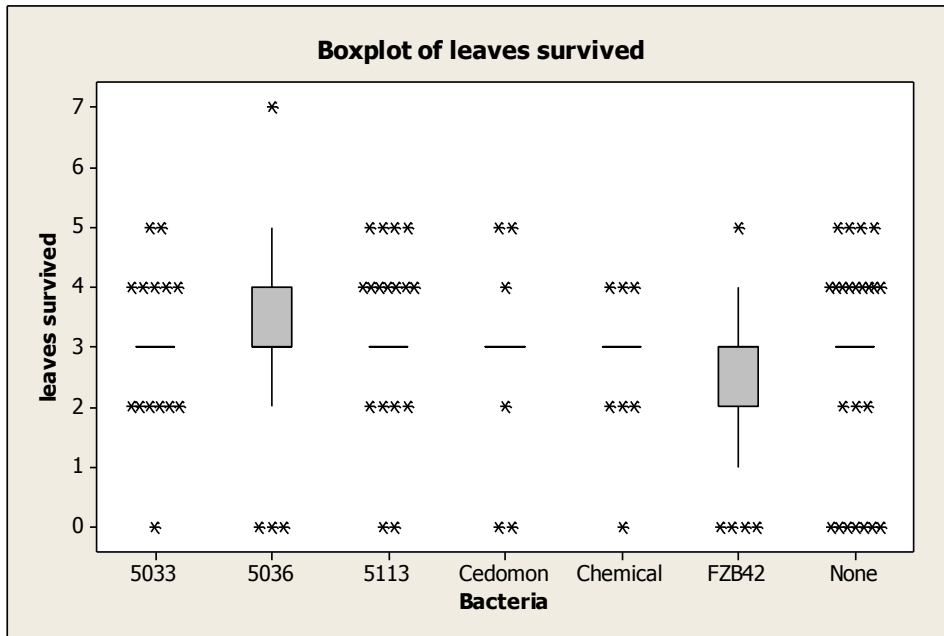


Fig 7. Box plot of leaf survival. Usually three leaves per plant survived. No statistically significant differences were found (n = 60, except for chemical and cedomon that were 30 and 20, respectively).

4.4 NANO ENCAPSULATION

No apparent effects were observed by the naked eye on the plants which had been treated with TiO_2 as compared to control. They had similar color, size, stem vigor and number of leaves (see fig. 8-10).



Fig 8. Belinda plants. Left pots were treated with TiO_2 and 5113, middle pots were treated with 5113 and to the right were untreated control.



Fig 9. Mitja plants. Left pots were treated with TiO_2 and 5113, middle pots were treated with 5113 and pots on the right were untreated control.



Fig 10. Vinjett plants. Left pots were treated with TiO_2 and 5113, middle pots were treated with 5113 and pots on the right were untreated control.

4.5 BACTERIAL SPORE GERMINATION AFTER NANO-TREATMENT

There was no statistically significant difference between the four treatments but there was a tendency towards a higher germination rate by the nano treatments. The nano-treated bacteria had more colonies after two days (see fig. 11).

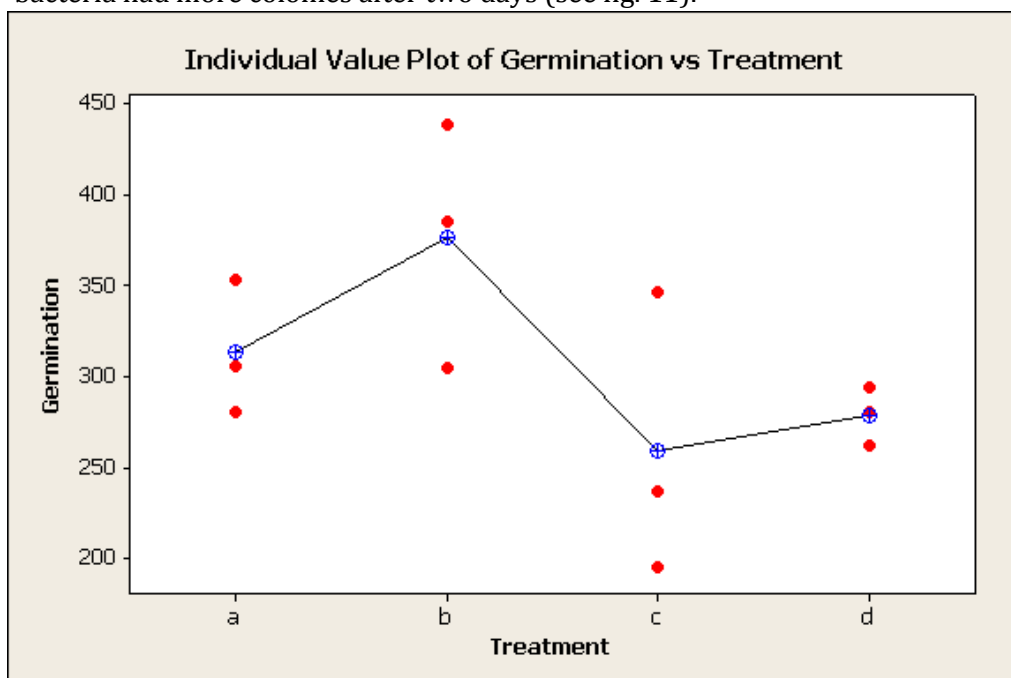
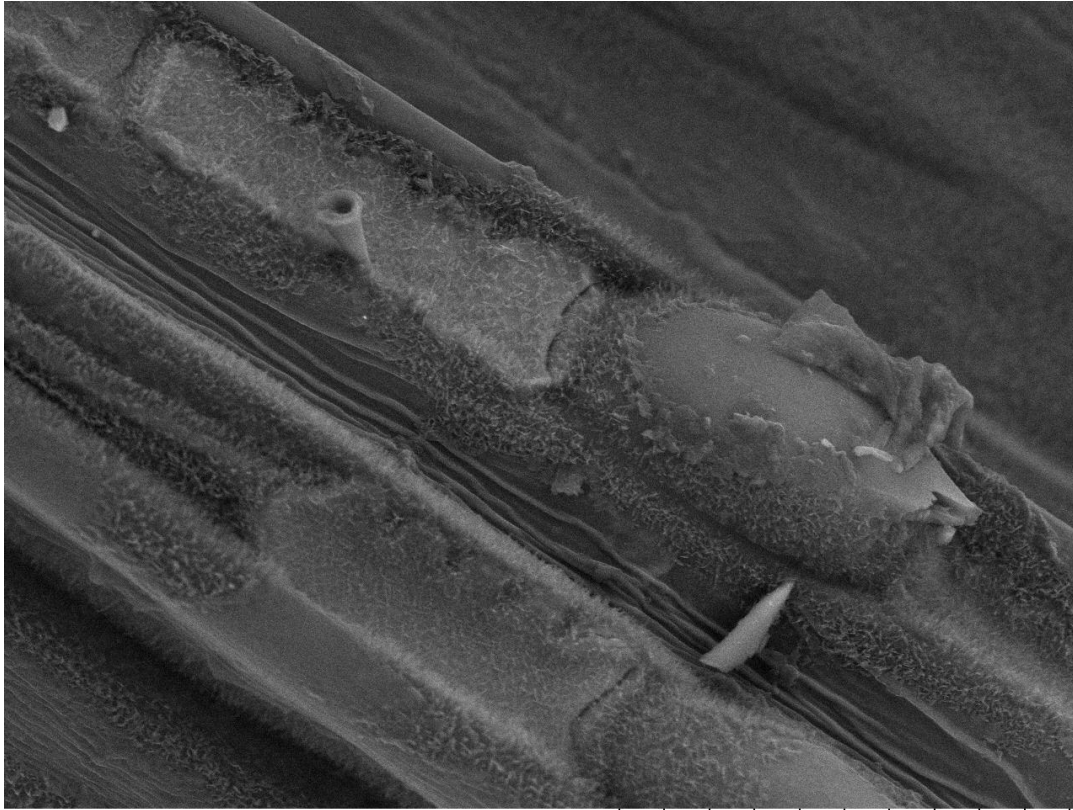


Fig 11. Number of colonies on LB-agar plates after 2 days; **a**: TiO_2 with MgCl_2 ; **b**: TiO_2 with water; **c**: 2% ethanol; **d**: with water. No statistically significant differences.

4.6 COLONIZATION

The study conducted with scanning electron microscope was not extensive enough to draw any conclusions about plant colonization by the bacteria. Three samples were studied: a 5 week old Tipple treated with 5113; a 10 week old Mitja treated with 5113 and a 12 week old Kerstin treated with 5113. Since no bacteria were found neither on roots nor on other tissues only a few example pictures are included here (see fig. 12-13).



TM-1000_1231

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15:45

L

D4.2

x1.5k

50 um

Fig 12. Bottom side of an oat leaf. A trichome can be seen to the right and to the left is possibly an ascocarp.

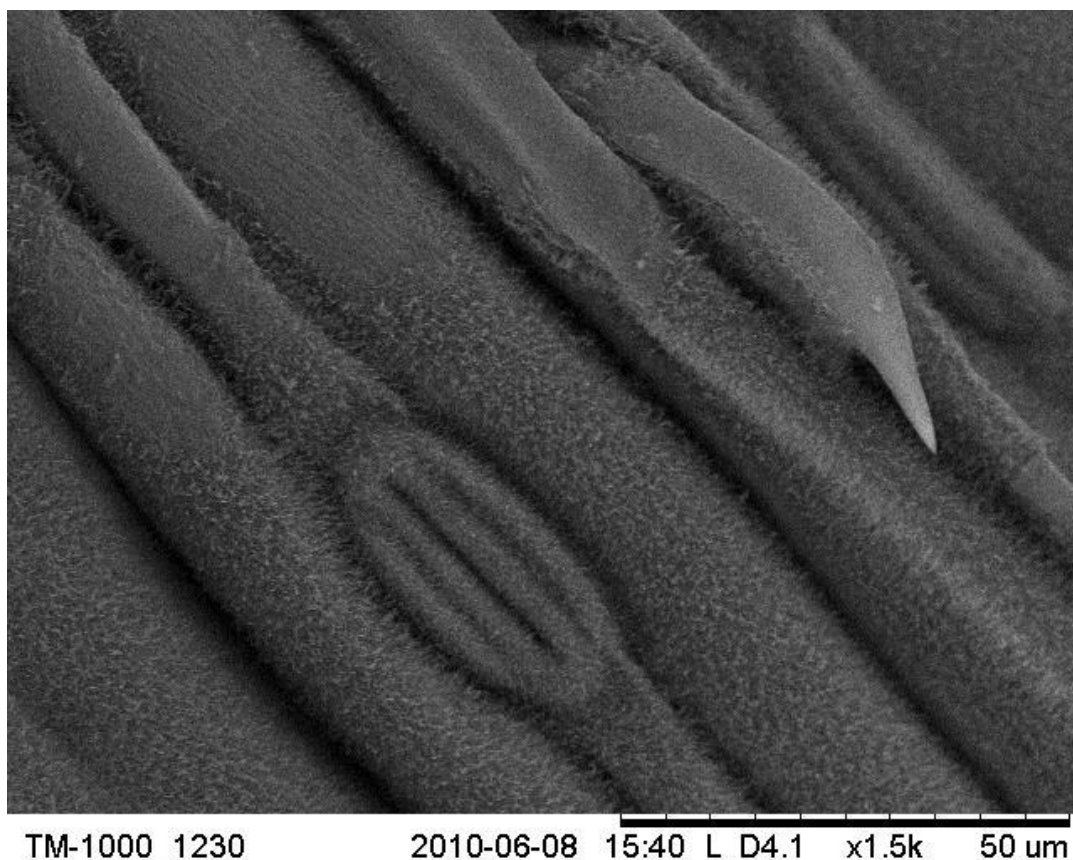


Fig 13. Lower side of an oat leaf. A stomata and a trichome can be seen.

5.0 DISCUSSION

5.1 PRIMING

The results indicate that the best overall bacterial strain to induce defense against cold stress in cereals is 5033. It showed good results with low standard deviation in chlorophyll content compared to the other treatments. In the statistical analysis including all species and treatments 5033 was the only one with statistically significant difference to the control. Although for wheat the best strain is FZB42, the negative effect on germination was not apparent and it clearly improved growth and stress tolerance. Strain 5036 did not show any successful results on any cultivar hence in future studies of freeze stress on cereals it could be excluded. It was the first time anyone has looked into the interactions between these *Bacillus amyloquelquefaciens* strains and cereals. Several cultivars and strains were chosen as an initial screening for beneficial interactions with cereals exposed to abiotic stress. The cultivars chosen are bred in Sweden and possess an inherent tolerance to cold temperatures. The bacterial strains represents *Bacillus* strains that have shown good results for protection of oilseed rape towards fungal pathogens (Danielsson et al., 2007) and also for abiotic stress tolerance on oilseed rape and wheat (Asari and Daim unpublished). Other

reports on successful and similar experiments are available, were for example Ashraf et al. (2004) studied salt stress with a strain of *Bacillus insolitus* on wheat and Pereyra et al (2006) and Creus et al (2004) studied effects of *Azospirillum* on wheat under osmotic stress and drought. The strains used in this investigation should also be tested under other abiotic stress conditions such as drought, heat and water logging. We know that ABA, which is produced by *Bacillus*, is involved in desiccation tolerance and cold tolerance in plants. Do the bacteria have some other mechanism to induce stress tolerance in plants? It should show in an investigation with water logging for example including a proteomic analysis. Unfortunately no molecular investigations have been conducted here but in the future it is important to investigate such factors to improve understanding on the mechanisms of these beneficial effects. It is also important to investigate not only plant vigor but also yield after priming. If the yield is reduced the treatment will be inutile. Woitke et al. (2004) found that *Bacillus subtilis* primed tomatoes exposed to saline stress produced lower yields than the unprimed control. It is also important to do field trials to see if the beneficial effects are still apparent under agricultural conditions. The field trials should be conducted on as many different soil types as possible since the bacteria are soil living and might have different effect in different habitats. Testing different application methods could also be interesting. For example spraying the bacteria on to the plants or maybe investigate how to get a stable population in the soil. The seed germination study should have been more thoroughly conducted and seeds should have been counted to show a more precise difference.

5.2 NANO ENCAPSULATION

It was an interesting observation that, as to the contrary to what one would expect, addition of the TiO₂ sol-gel actually seemed to increase the number of colonies on LB-agar plates. There was no statistically significant difference but since it was such a small pilot study a repetition with more replicates may reveal a statistically significant difference. If it is so that the TiO₂ increases survival of the bacteria in an optimal environment in vitro it will probably also be beneficial in the field. Hence field experiments should be conducted to see if this can increase the beneficial effect of the bacteria. The plants induced with nano-encapsulated bacteria did not show any difference to control in the controlled environment of growth chambers but in the field various other factors affect both bacteria and plants. This was the first time a TiO₂ sol-gel has been tested on a *Bacillus* strain for this purpose and time was limiting since suitable experimental conditions had to be tried out. In the future more extensive studies should be made. For example 2d-gel electrophoresis should be conducted to investigate protein expression that could reveal the physiological response of the bacteria and identify proteins that support germination seemingly stimulated by the preliminary experiments conducted. Further, if there is any increased levels of reactive oxygen species due to nanoparticles, as suggested by studies of other systems, could be analyzed as reflected e.g. in various antioxidant enzymes.

5.3 COLONIZATION

Use of a scanning electron microscope is quite time consuming and there was not enough time during this project to make a conclusive investigation. For example a suitable method

of protecting the biological material to avoid deterioration during observations had to be found. To see bacteria with the approximate size of 2-5 μm one needs to use at least 1000 times magnification hence the area to be investigated is very big. The mere 20 hours I spent on SEM was not sufficient to draw any firm conclusions about *Bacillus* colonization. It appears though that the *Bacillus* bacteria do not colonize the phyllosphere to any significant extent but are probably more abundant in the rhizosphere. Unfortunately, root tissues from soil grown plants are very difficult to prepare for SEM without losing loosely attached bacteria due to the extensive washing needed.

CONCLUSIONS

Some of the investigated strains do confer increased tolerance to freeze stress in cereals and it would be interesting to investigate into the mechanisms of the effect. Further investigations into other abiotic stresses with these bacteria is also interesting. The effect of nano-encapsulation is yet to be proven and should also be investigated more thoroughly.

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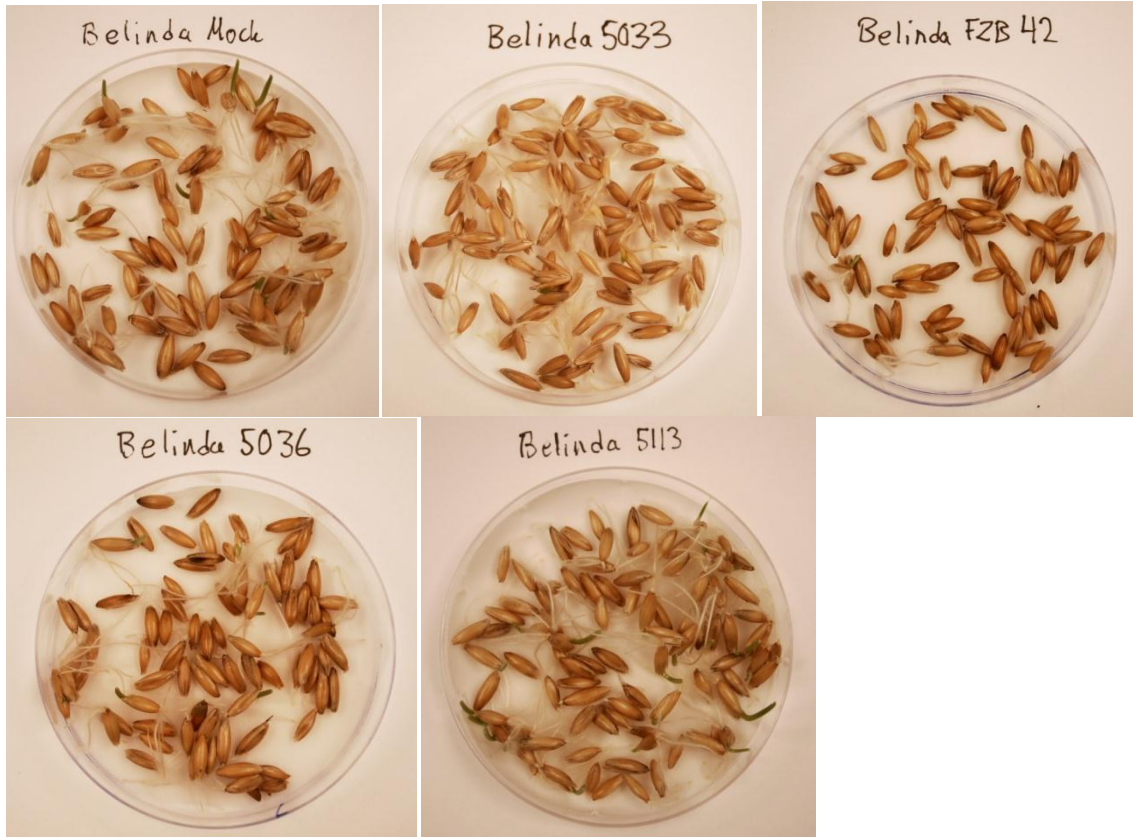
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7.0 APPENDIX 1

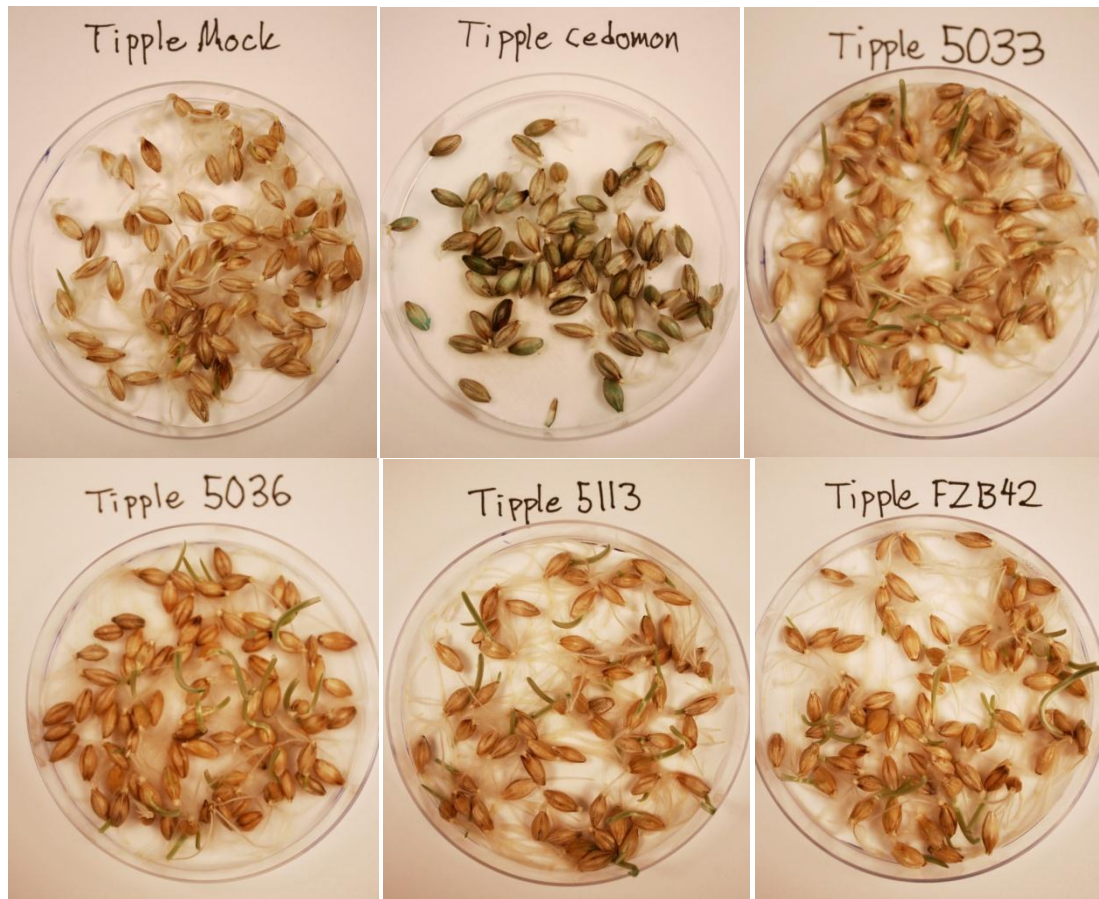
BELINDA SEED GERMINATION



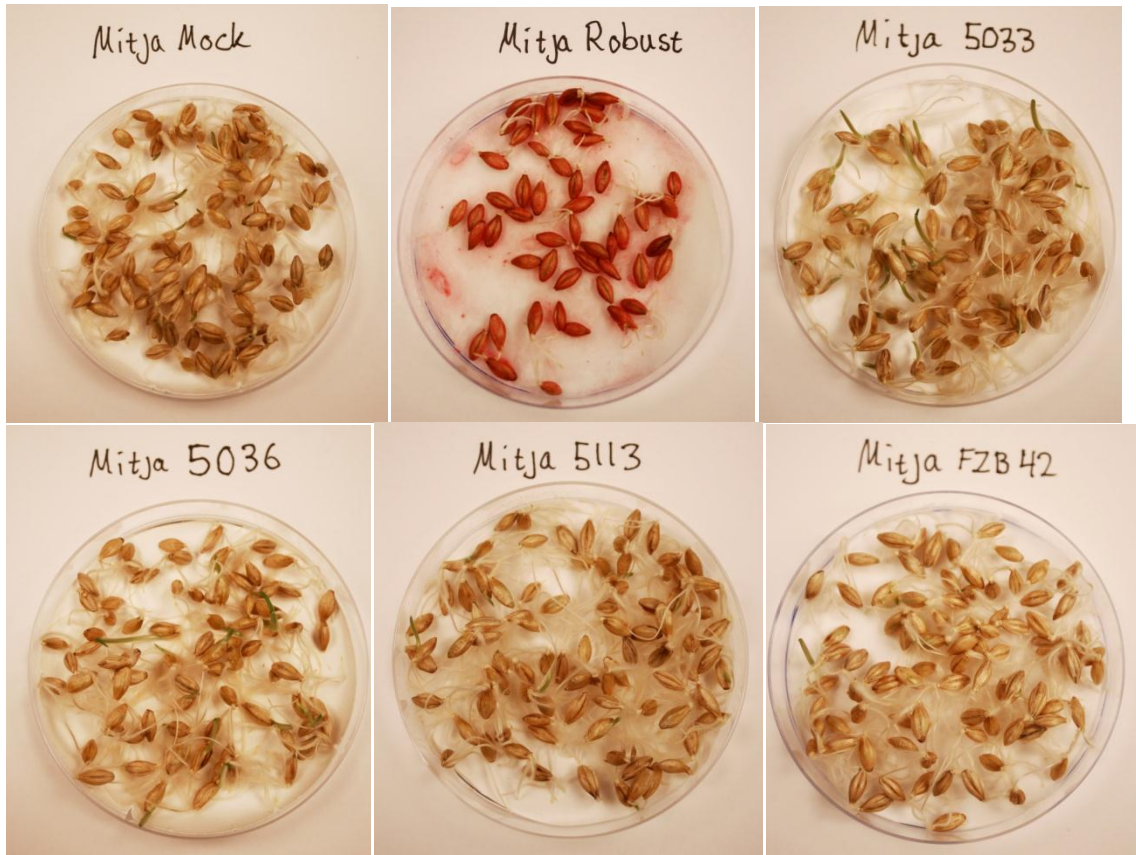
KERSTIN SEED GERMINATION



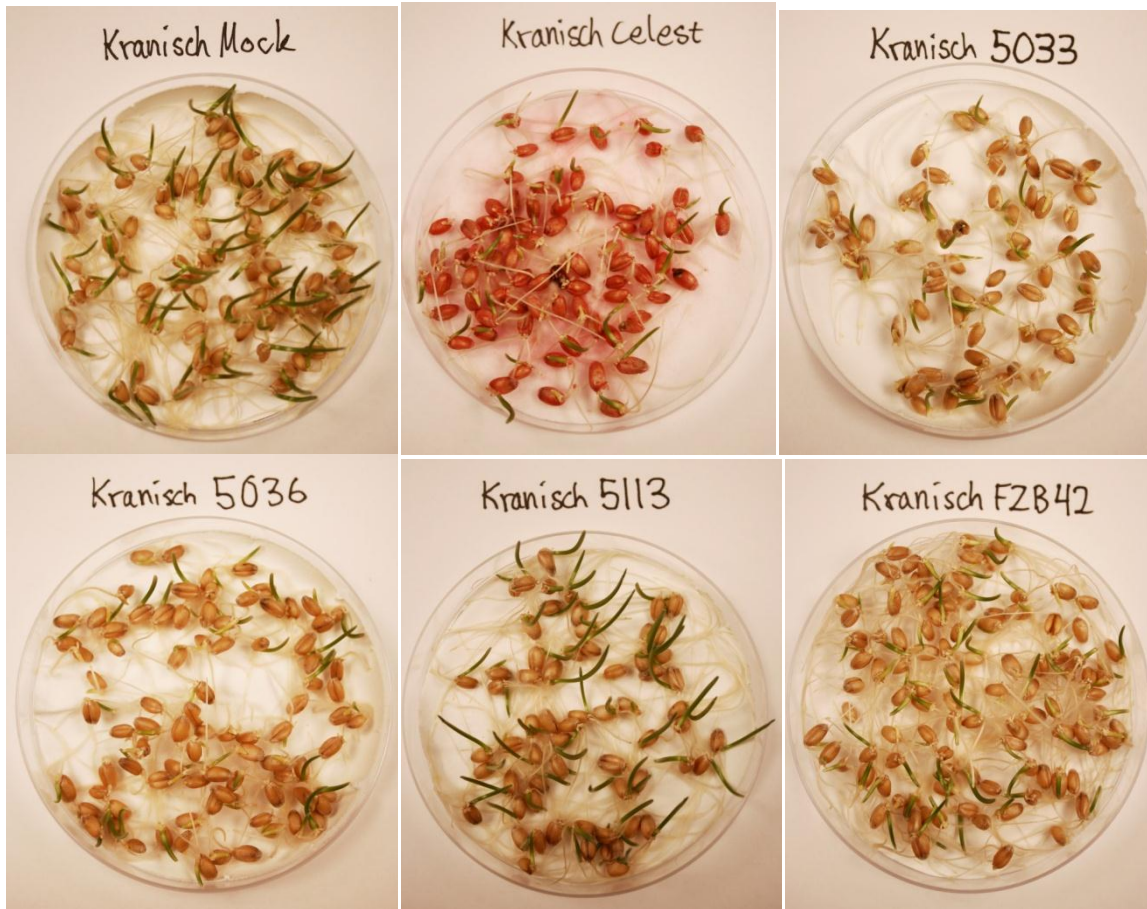
TIPPLE SEED GERMINATION



MITJA SEED GERMINATION



KRANISCH SEED GERMINATION



VINJETT SEED GERMINATION

