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Final degree project

Evaluation of the efficacy of *Bacillus amyloliquefaciens* against *Ditylenchus angustus* infection in rice

Joan Ignasi Feixas Maneu

Degree in Biotechnology

Tutor: Dr. Jordi Planes Cuchi. Professor in Universitat de Vic, Spain.

Exchange tutor: Dr. Tina Kyndt. Research professor in University of Gent, Belgium.

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1.- ABSTRACT

Title: *Evaluation of the efficacy of Bacillus amyloliquefaciens against Ditylenchus angustus infection in rice*

Keywords: bacteria, nematode, infection, rice, plant growth.

Author: Joan Ignasi Feixas Maneu

Supervisors: Dr. Tina Kyndt (University of Gent) and Jordi Planes Cuchi (Universitat de Vic).

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The rice stem nematode *Ditylenchus angustus* causes "Ufra" disease in rice and causes substantial yield losses. Environmental side effects associated with chemical control of this nematode underline the urgent need for alternative environmental-friendly strategies. Antagonistic bacteria have been shown to be promising microorganisms for the biological control of plant-parasitic nematodes. However, there is little information about using bacteria to reduce the *D. angustus* infection in rice. We isolated and identified some bacterial strain as *Bacillus amyloliquefaciens*. It has been shown that man strains of plant-associated *Bacillus amyloliquefaciens* stimulate plant growth and produce secondary metabolites that suppress plant pathogens. The aim of this research is to evaluate the efficacy of our identified *B. amyloliquefaciens* against *D. angustus* infection in rice.

2.- INTRODUCTION

Rice, *Oryza sativa L.* is one of the most important cereal grains since it is consumed by a large part of the human population, over 3 billion people globally (FAOSTAT, FAO Statistics Yearbook 2009, 1). *Oryza* is a member of the grass family (*Poacea*). There are only two species cultivated, *Oryza sativa L* and *Oryza glaberrima*. In our study, we used *Oryza sativa*. Moreover, *Sativa* has two subspecies, *indica* and *japonica*. The first one is prevalent in tropical regions, and *japonic*a mostly in temperate regions of East Asia (Van Bockhaven, 2014).

In our studies, *Oryza sativa subsp. Japonica* was decided to be used, since it is an attractive model plant for monocotyledons with a tremendous amount of genomic and molecular information available, such as the Rice Genome Research Program (International Rice Genome Sequencing Project, 2005).

Rice can be grown in different climatic conditions and it can be grown either in dry or wetland conditions, even at low or high altitudes. For example, in Asia, rice is grown in fields that are permanently inundated, mostly by irrigation. (Van Bockhaven, 2014). Because of this amount of water, some pests, like nematodes can infect the rice plants. These nematodes can affect growth and yield of the crop. Nematodes can be foliar or root parasites (Bridge & Starr, 2007). Nematodes are spread worldwide, but in tropical and subtropical regions they tend to have a big impact, basically because these regions depend on rice as the primary food source.

Even the use of hazardous chemicals could help to solve the problem, rice producers believe that these practices are environmentally undesirable as well as expensive and are starting to consider other options than the release of a new variety of pesticide (De Vleesschauwer, 2008). Our research goes through that direction, aiming to develop new disease control strategies which are environmentally safe, using a bacterium, *Bacillus amyloliquefaciens*, as a bio pesticide to solve the nematode problem in rice plants.

2.1.- The nematode, *Ditylenchus angustus*

Nematodes are microscopic worms, among which some can parasite plants. We find two types of nematodes: endoparasitic and ectoparasitic nematodes. The first ones spend part of their life cycle within the host plant, and ectoparasitic do not enter the plant tissue, they can stay in the soil, feeding from plant roots (Haegeman et al., 2012).

The nematode *Ditylenchus angustus* is native to Asia and causes one of the most important diseases affecting rice, Ufra. D. angustus is an ectoparasitic nematode that feeds on young foliar tissues, in the meristem of the leaf collar, the panicles and the seeds (Bridge J. et al., 2005). Nematodes are able to migrate from one plant to another by stem and leaf contact or under high humidity, making it easy to infect all the crop. The infection produces several symptoms. In the vegetative growth stage the main symptom is leaf chlorosis. There will be white patches or speckles in a splash pattern at the bases of young leaves. Necrotic brown stains may develop on leaves and leaf sheathes. Depending on the severity of the infection, the chlorotic leaf areas, the tiller or the whole plant can turn brown or even die (Bridge & Starr, 2007).

When an infection takes place, the host cell wall softens and can be degraded as well. PNN (Plant parasitic nematodes) can suppress or avoid host defence and can manipulate the host signalling pathways. The stylet, a needle-like structure, is responsible for the infection. It damages the rigid plant cell wall during migration through the plant tissue. Moreover, the stylet is responsible for delivering secretion in host tissue, a cocktail of effectors to degrade and soften the cell wall, degrading cellulose, hemi-cellulose or pectin. Nematodes also secrete a variety of proteins from the stylet but without hydrolytic activity, such as expansins, proteins that promote the action of cell wall degrading enzymes (Haegeman et al., 2012).

As mentioned before, plant diseases cause important crop losses in production and storage as well. Nowadays, cultivators still rely on chemical pesticides to solve this problems. However, the continuous utilization of these pesticides can cause environmental contamination and presence of pesticide residues on food. That's why, a new alternative is

emerging, the use of natural antagonistic microorganisms. They present many advantages in terms of sustainability, mode of action and toxicity compared with chemical pesticides (Cawoy et al., 2011). These bio pesticides are based on endophytic bacteria (Reva et al., 2004). An endophytic bacteria, is any bacteria able to colonize the interior of plants. They can be active and latent pathogens (James & Olivares, 1998). Endophytic bacteria have been isolated from both monocotyledonous and dicotyledonous plants (Brooks et al., 1994) and they can be either Gram-positive or Gram-negative (Kobayashi, D.Y. and Palumbo, 2000)

2.2.- The bacteria, *Bacillus amyloliquefaciens*

In our research, we used *Bacillus amyloliquefaciens*, a gram-positive bacterium, a plant-associated species belonging to the family *Bacillaceae* (Niazi, et al., 2013). *B. amyloliquefaciens* has the ability to simulate plant growth and also suppress plant pathogens (Chen et al., 2007). It has been shown that strains of *B. amyloliquefaciens* and *B. subtilis* can synthesize plant growth promoting substances such as gibberellins and indole-acetic acid (Turner & Backman, 1991), extracellular phytase (Idriss et al., 2002), chitinase (Wang et al., 2002) and antifungal peptides (Pinchuk et al., 2002).

Plant growth promoting rhizobacteria (PGPR) can be defined as root-colonizing bacteria that exert beneficial effects on plant growth and development (Choudhary & Johri, 2009). The rhizosphere is the narrow zone of soil influenced by the root system. This zone is rich in nutrients, such as amino acids and sugars, providing a rich source of energy and nutrients for bacteria as well as for the plant (Dobbelaere et al., 2003), and PGPR colonize the root surface and the closely adhering soil interface, the rhizosphere (Choudhary et al., 2007).

Plant growth promoting rhizobacteria (PGPR) can affect plant growth in two ways, directly or indirectly. In the first scenario PGPR can fix atmospheric nitrogen and supply it to plants for example. On the other hand, entophytic bacteria, as well as PGPR, lessen or prevent the effects of phytopathogenic organisms, and this ability can be considered to be an indirect promotion of plant growth (Lodewyckx et al., 2002).

PGPR can elicit plant defence, named induced systemic resistance (ISR). Once the plant is treated with a PGPR, the host defence is elicited and a reduction in the severity of the disease can be noticed. (Kloepper et al., 2004).

We are not certain whether *Bacillus amyloliquefaciens* has an endophytic effect or a PGPR. In practice, the difference between endophytic bacteria residing in the root and bacteria colonizing the rhizosphere is often determined by the surface sterilization technique applied (Lodewyckx et al., 2002).

2.3.- Systemic plant resistance

Plants have different resistant pathways regulated by different genes. There are two types of systemic resistances, the acquired defence (SAR) and the induced defence (ISR). In both cases, plants defence pathways are triggered by a prior infection (SAR) or treatment (ISR) that results in resistance against pathogens or parasites (Choudhary et al., 2007). It is believed that PGPR can elicit ISR, in our experiments a comparison between ISR and SAR we'll be carried out by qPCR to check whether *Bacillus amyloliquefaciens* can activate ISR.

SAR can be triggered by exposing the plant to virulent, antivirulent and non-pathogenic microbes. On the other hand, ISR is the process where the plant elicits a host defence activated by an environmental stimuli or a PGPR (Vallad & Goodman, 2004).

2.4.- Defence pathways

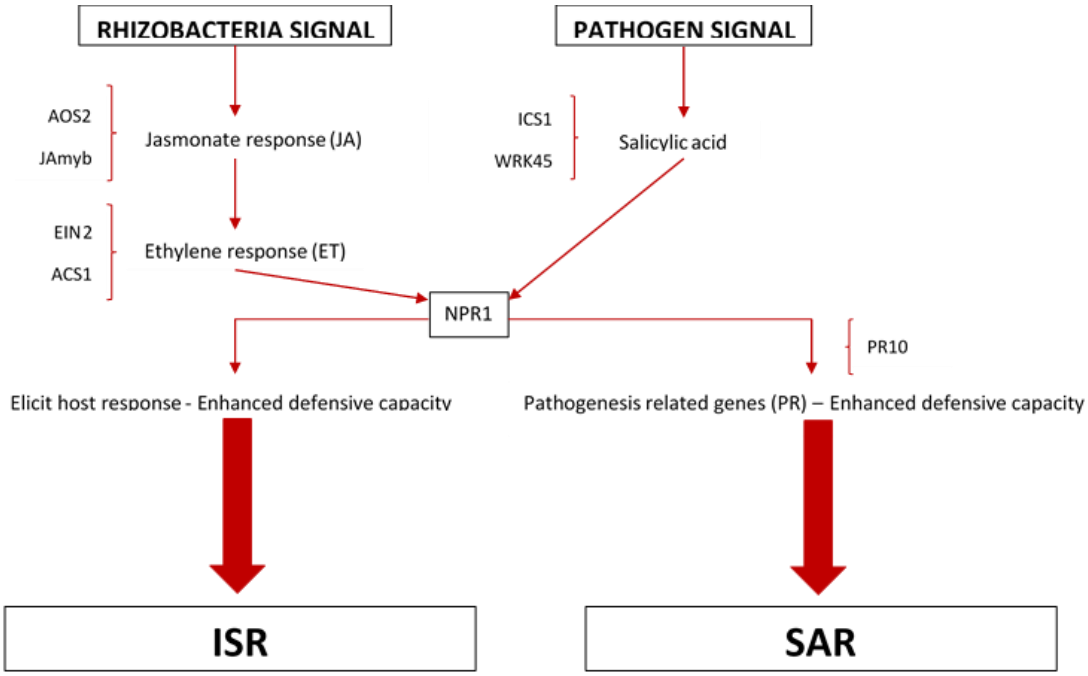


Fig. 1. Genes chosen for qPCR analysis are shown in the figure. ISR and SAR pathways are represented depending on the plant attack. Depending if it is a rhizobacteria or a pathogen, different defence pathways will be activated.

A network of interconnected signalling pathways regulates induced defence in plants against pathogens. As shown in figure 1, the primary components of this network are salicylic acid (SA), jasmonate (JA) and ethylene (ET) (Choudhary & Johri, 2009).

Different genes were chosen for each pathways to check the relative expression in different conditions (inoculation with bacteria, infection with nematodes and infection with nematodes when the plant is inoculated with the bacteria). ICS1 and WRKY45 were chosen for SA signalling pathway. SA is involved in the process providing SAR, protecting the plant from further infection after an initial pathogen attack (Beckers & Spoel, 2006), which accumulates in plants prior to the onset of SAR (Dong, 1998). An accumulation of SA in the plant leads to the activation of pathogenesis related genes (PR genes) ending in a SAR activation.

Nahar et al., 2011, conclude in their article "The jasmonate pathway is a key player in systemically induced defence against root knot nematode in rice", that ET-induced systemic defence involves ET signalling and a strong activation of JA biosynthesis and signalling genes. These results indicate that JA pathway is a key defence pathway involved in root knot nematodes resistance of the rice root system. However, SA pathway has a minor positive effect on root defence against root knot nematodes.

Moreover, several studies have shown that SA can induce PR genes, for example, Durner, Shah, & Klessig, 1997, used salicylic acid in tobacco to induce PR genes expression, and as a consequence, the plant enhanced resistance against tobacco mosaic virus. Moreover, they stated that SA in an endogenous compound, operating in the signalling pathway for plant defence. Even though it is known the effect of an accumulation of SA in the plant, the mechanism of SAR induction is still unclear (Durner et al., 1997).

PR10 is one of the genes involved in plant defence. PR proteins have special properties, allowing them to help the plant to resist against acidic pH and proteolytic cleavage, and consequently survive in harsh environments (Stintzi et al., 1993). PR10, belongs to a group of PR genes, PR1, but the biological activity of the proteins expressed by the gene is still unknown, even though some members have been shown recently to have antifungal activity. It is believed that PR genes are overexpressed during the response of plants to infection by viruses, viroids, bacteria or fungi. However, PR genes can be overexpressed by chemical treatments, treatments with phytohormones, osmotic stress and salt stress conditions (Grosset et al., 1990). An overexpression of PR genes are expected after treating the plants with nematodes and bacteria.

As mentioned above, ISR is potentiated by PGPR, such as, *Bacillus amyloliquefaciens* and doesn't involve the accumulation of PR or SA (Pieterse et al., 1996) but relies on pathways regulated by JA and ET (Knoester et al., 1999). AOS2, JAmyb and EIN2, ACS1 genes involved in JA and ET respectively. Their signalling pathways influence each other in a complex network of synergistic and antagonistic interactions (Koornneef & Pieterse, 2008).

Evidence strongly suggests the importance of JA and ET as alternative signals in the induction of resistance against microbial pathogens (Dong, 1998).

Another gene involved in plant defence is NPR1. NPR1 has a key role in plant defence. Cao et al., 1994, discovered in their research with *Arabidopsis*, that plants containing mutations at NPR1 locus are compromised in their ability to activate efficiently SAR and ISR. Moreover, transgenic plants expressing lesser amounts of NPR1 are also more susceptible to infection from pathogens

The investigation is divided in two big studies, seed treatment study and soil treatment study. Every treatment will be inoculated by different bacteria concentrations. Then, the plants will be inoculated with nematodes so we will be able to collect data after several days and discuss whether the bacteria have an effect on an induced systemic resistance against the nematode or not. In parallel we will carry a growth promotion of rice without the nematode inoculation, being able to see what effects the bacteria have on the plant in different aspects, such as root and shoot length or root and shoot weight. A qPCR analysis will be also carried out in order to verify if the genes involved in defence pathways are downregulated or upregulated in different stress conditions.

In this report, the efficacy of *Bacillus amyloliquefaciens* against *Ditylenchus angustus* infection in rice will be evaluated as well as the efficacy of *Bacillus amyloliquefaciens* as a plant growth promoter rhizobacteria.

3.- OBJECTIVES

1. To examine the activity of *B. amyloliquefaciens* (Ba) in growth promotion of rice at different bacterial concentrations.

2. To investigate Ba induced systematic resistance against *D. angustus* (Da) in rice at different bacterial concentrations.

3. To analyse the expression of some defence related genes in Ba-rice-Da interaction.

4.- MATERIALS AND METHODS

- **Culture and bacteria**

We used solid LB medium [Agar (Sigma-Aldrich) + Luria-Bertrani broth (Sigma-Aldrich) + distilled water)] to culture the bacteria. The bacterium, *Bacillus amyloliquefaciens* (*Ba*W1) needs to be without light and at 30ºC for 1-2 days in order to grow. *Bacillus amyloliquefaciens* was isolated from the roots of wheat from the field of Gazipur, district of Bangladesh.

To develop the experimental setup, bacterial suspensions at different concentrations were prepared by scraping off the growth plates and suspending bacteria in sterile saline solution (0.85 % NaCl). Densities of the bacterial suspensions were adjusted to the desired concentrations (10^4 cfu/mL, 10^6 cfu/mL, 10^8 cfu/mL) with optical density at 620 nm (Han & Lee, 2005).

- **Seed pre-treatment**

Seed treatment assay was performed with dehisced rice seeds (*Oryza sativa* cultivar "Nipponbare", japonica type; provided by USDA; GSOR-100). The procedure to sterilize the surface of the seeds consisted in three steps. First, the surface of the seeds was treated with 70 % ethanol for 1 minute; next, seeds were kept for 5 minutes in a 0.1 % Mercuric Chloride solution; and finally they were washed 5 times with sterile distilled water

- **Growth promotion studies**

The main objective of this study was to evaluate the influence of *BaW1* on the development of health of *O. sativa*. In order to stimulate an association between the bacterium and the plant we compared two methods which will be referred to as the seed treatment and the soil treatment respectively. In the seed treatment, surface-sterilized seeds were pre-incubated with bacterial suspensions at different concentration while in the soil treatment surface-sterilized seeds were planted in a substrate with different concentrations of bacteria.

In seed treatment 30 seeds were submerged for 30 minutes in 10 mL suspensions at different bacterial concentrations (10^4 cfu/mL, 10^6 cfu/mL, 10^8 cfu/mL) and later dried under laminar air flow. The 30 dried seeds were placed in sterile wet paper sealed petri dishes at 30ºC without light for 5 days in different plates, depending on the concentrations, see figure 2. 30 seeds were used as controls and were submerged for 30 minutes in 10 mL of sterile saline solution before transfering to a sterile wet paper sealed petri dish. The seed germination percentage was calculated before transplanting the seeds to autoclaved SAP (Reversat et al., 1999) in a glass tube (15x2.5 cm). Tubes were stored at 26ºC under a 12h/12h light regime (150 µmol/m^2 /s) (Thimijan & Heins, 1983) and 70-75% Relative

Humidity as before. After 28 days of sowing, root and shoot length and weight were measured.

In the soil treatment, which was performed in parallel to the seed treatment, approximately 80 surface-sterilized seeds were placed in sterile wet paper sealed petri dish at 30 ºC without light for 5 days. 500 µl of bacteria were drenched over SAP-substrate at concentrations of 10^4 cfu/mL, 10^6 cfu/mL, and 10^8 cfu/mL during seedling transfer. Tubes were kept at 26ºC under a 12h/12h light regime (150 µmol/m^2 /s) and 70-75 % relative Humidity.

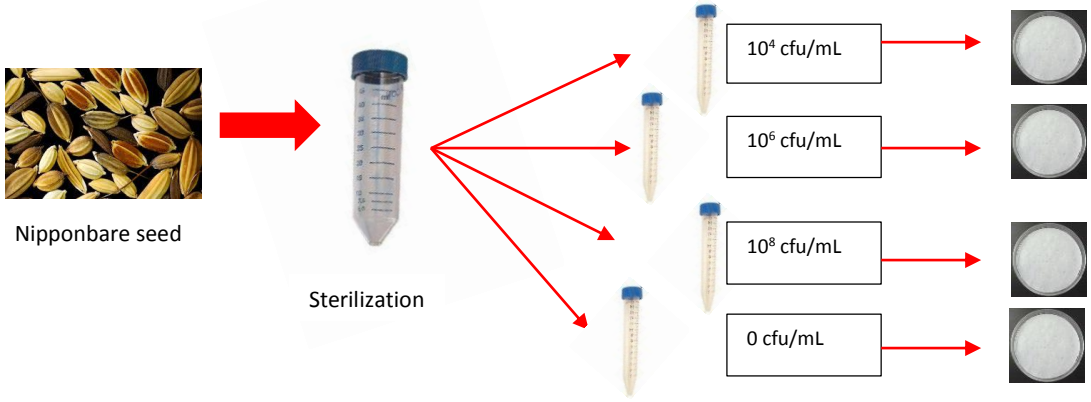


Fig. 2. Seeds were first surface sterilized. Seeds were then submerged bacterial suspensions at different concentrations for 30 minutes. Once the seeds were dried, they were placed in sterile wet paper sealed petri dish at 30ºC without light for 5 days

After 28 days of sowing, root and shoot length and weight were measured. Plants were first washed in order to remove the remaining sand in the roots. Seeds were removed from the plant. Then, root and shoot were separated. Root and shoot length were measured in centimetre using a ruler. Afterwards, the root was wiped away with tissue papers to remove excess water, and weight was measured in milligrams using analytical balance. Shoot weight was measured using an analytical balance as well.

- **Root colonization by *B. amyloliquefaciens***

To evaluate the endophytic colonization level the roots of three plants from each experimental condition were collected, washed and weighed. After surface sterilization (70 % ethanol for 1 minute, next, seeds were kept for 5 minutes in a 0.1 % Mercuric Chloride solution), 1 g of roots were macerated using SDW (sterile distilled water), followed by dilution plating on solid LB medium and incubated at 30ºC for 2 days. The number of colony forming units per gram of root weight was determined.

To evaluate the epiphytic colonization three roots from the evaluation of the endophytic colonization test were directly plated in LB medium.

- **Nematode growth**

The nematode, *D. angustus* was collected from the plant pathology division of BRRI, Gazipur-1706. The nematode was originally isolated from an infested farmer's field from Gazipur district, Bangladesh. The nematode culture was maintained in vivo on the susceptible rice cultivar BR3 (indica type; provided by BRRI) at 26°C under a 12h/12h light-regime (150 µmol/m²/s) and 70-75% relative humidity and in vitro by rice plantlet culture (Richard, 1994) in Petri dish, maintained in an incubator at 25ºC with a 12 h photoperiod. Nematodes were extracted from *D. angustus* infected stem using the modified Baermann method (Luc M, Sikora RA, 2005). Several infected nematode stems were cut into small pieces and spread on the top of the water. The nematode suspension was collected and nematodes were counted under a light microscope.

- **Infection protection studies**

To evaluate whether the symbiosis between the plant and the bacterium has a protective effect against nematode infections, fifteen day old rice plants grown under the different condition specified in the soil treatment set up and control were inoculated with approximately 100 nematodes of *D. angustus* per plant (as described by Rahman, 1993). 15 days after sowing, the water level was raised up to the upper most node of the seedling. The infection level of the plants was evaluated 15 days post inoculation (dpi) by counting the number of nematodes per plant. For counting the nematodes, each plant was cut into 5mm pieces and placed over a sieve overnight to release nematodes from the plant tissues. Later, water was collected and placed in a petri dish in order to count manually under a light microscope.

- **RNA extraction, cDNA synthesis and qRT-PCR**

Shoot RNA was extracted using RNeasey Plant MiniKit (Qiagen) following the manufacture's protocol. RNA concentration and purity was measured using NanoDrop 2000 spectephotometre (Thermo Scientific). The DNAse treatment was carried out with 1.8 µl buffer with $MgCl_2$ (DNAse I + $MgCl_2$), 1 µl of RNAse inhibitor (Ribolock RI), 1 µl of DNase I in a total volume of 18 µl. The mixture was incubated at 37 ºC for 30 minutes after which 2µl 25 mM EDTA was added and incubated for 10 minutes at 65ºC to stop the reaction.

cDNA synthesis was done adding 1 µl oligo dT, 2 µl 10 mM dNTPs and 4 µl RNAse-free water to the DNAse-treated RNA and then incubated for 5 min at 65 ºC (allows to remove secondary structures). Later on, 8 µl 5X first strand buffer (Invitrogen) and 4 µl 0.1 M DTT were added to the mix, followed by 2 minutes of incubation at 42 ºC. Finally, 1 µl of SuperScript II Reverse Transcriptase (200U/µl; Invitrogen) were added and placed it at 42 ºC for 2 hours. The quality of cDNA was tested by performing a standard RT-PCR with two reference gene (*Os*EXP, *Os*EXP Narcai) and checking the products on a 1.5 % agarose gel.

The 'qPCR Core Kit for SensiMix SYBR® No-ROX (Bioline) is provided as a 2x mastermix and was used in all QRT-PCR analyses. All PCR reactions were performed in 3 technical replicates. Two independent biological replicates, each containing a pool of 4 plants, were analysed. The reactions were performed in the Rotor-Gene 3000 (Corbett research) using Rotor Discs (72, Qiagen) and results were generated by the Rotor-Gene 6 software. PCRs were performed under following conditions: 45 cycles of [25" at 95ºC, 40" at 58ºC and 20" at 72ºC]. After the PCR reaction, a melting curve was generated by gradually increasing the temperature to 95 ºC to test the amplicon specificity. Data was analysed using the REST 384 software (Corbett Research; (Pfaffl, Horgan, & Dempfle, 2002)). This software uses a permutation analysis to compare the relative expression between a sample and control group and to determine the statistical significance of the results.

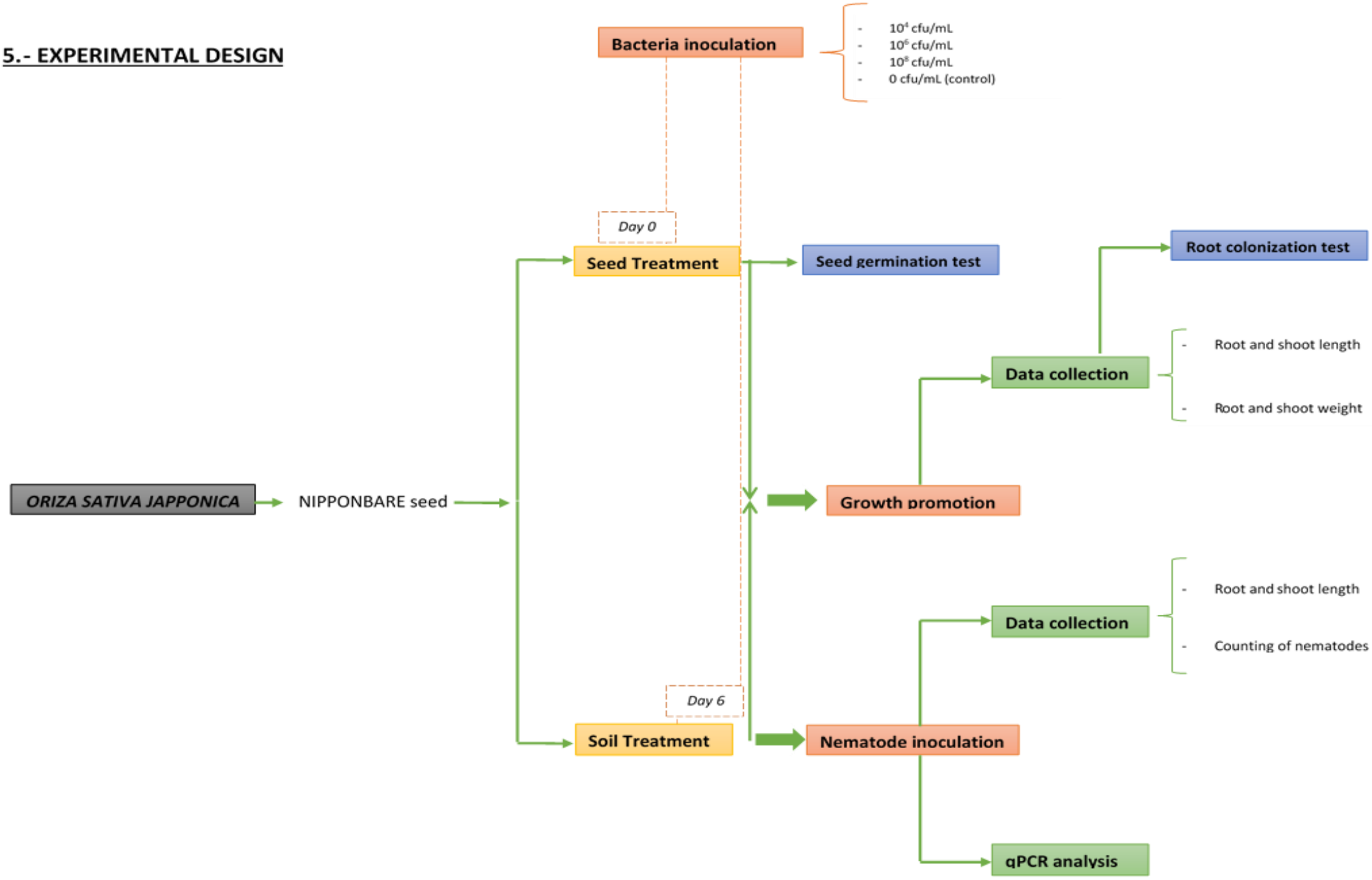
Target Gene	**GenBank Accession/Locus no.**	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
OsEXP	LOC_Os03g27010	TGTGAGCAGCTTCTCGTTTG	TGTTGTTGCCTGTGAGATCG
OsEXPNarcai	LOC_Os07g02340.1	CACGTTACGGTGACACCTTTT	GACGCTCTCCTTCTTCCTCAG
OsEin2	Os07g06190	GCGCATGTTGTAGAAGACGA	CAGGCAGCTTCGAATCAAGT
OsACS1	LOC_Os03g51740	GATGGTCTCGGATGATCACA	GTCGGGGGAAAACTGAAAAT
OsJAmyb	AY026332	GAGGACCAGAGTGCAAAAGC	CATGGCATCCTTGAACCTCT
OsAOS2	NM_001055971.1	TGCGCGACCGCCTCGATTTC	GGCCAGGCGGGACGTTGATG
OsWRKY45	Os05g0323900	AATTCGGTGGTCGTCAAGAA	AAGTAGGCCTTTGGGTGCTT
OsICS1	LOC_Os09g19734	TGTCCCCACAAAGGCATCCTGG	TGGCCCTCAACCTTTAAACATGCC
OsPR10	Os07g0418500	TCGTATGCTATGCTACGTGTTT	CACTAAGCAAATACGGCTGACA

Table 1. Summary of the reference and target genes used in the study, with their GenBank accession number or locus number, and the primer pair used for qRT-PCR

- **Data collection and statically analyses**

All statistical analyses were performed in SPSS. Normality of the data was checked by applying the Kolmogrov-Smirnov Test of Composite Normality (=0.05). Collected data was analysed using ANOVA. The means of the control and treated group were compared by Duncan's multiple mean comparison test.

5.- EXPERIMENTAL DESIGN



6.- RESULTS

6.1.- Growth promotion activity of *Bacillus amyloliquefaciens* (BaW1)

We studied the effect of W1 isolate, identified as *Bacillus amyloliquefaciens* on shoot and root growth, measuring root and shoot weight and length at 5 days after seed inoculation or soil drenching.

- *Seed germination*

The bacteria treated rice plants didn't have any effect on seed germination as no significant differences were observed between treatments and control. However, there seems to be a tendency to a negative correlation between the germination success and the bacterial concentration.

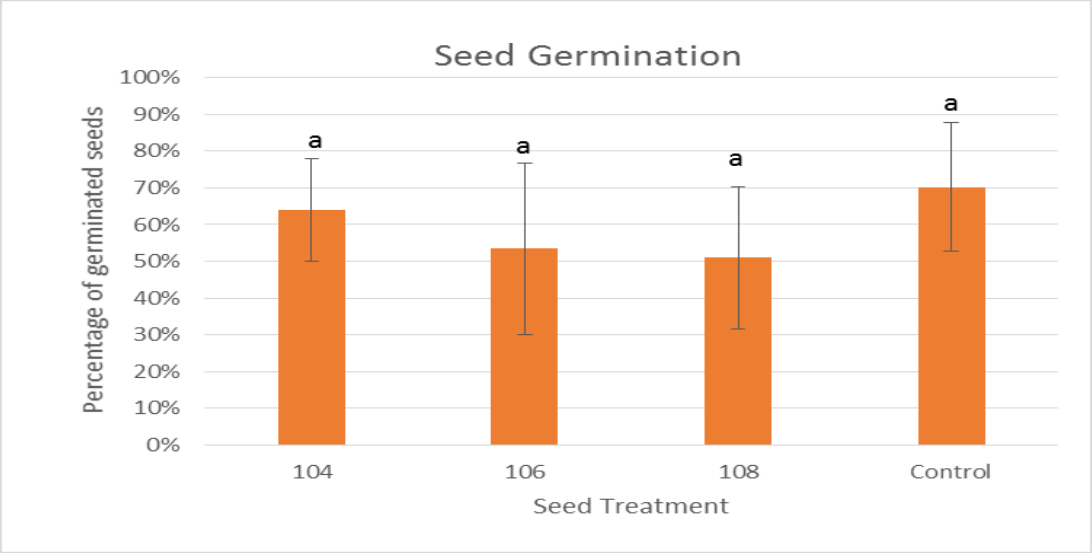


Fig. 3. Effect of *B. amyloliquefaciens* at different concentration on seed germination of Nipponbare seeds after 5 days of incubation. The columns represent the mean and standard error of seed germination recorded on 30 seeds in 7 different experiments. No significant differences are observed with the different treatments. Different letters indicate statistically significant differences (Duncan's multiple range test with $\alpha = 0.05$).

- *Shoot and root length*

The BaW1 treated rice plants increased shoot and root length in almost all the treatments compared to the control plants but the increase was not statistically significant.

The shoot length (Fig. 4) was highest in the soil treatment with 10^4 cfu ml^{-1} although the results are not significant. The lowest bacterial concentration also had a growth promoting effect in seed treatment compared with control, but again, the differences were not significant.

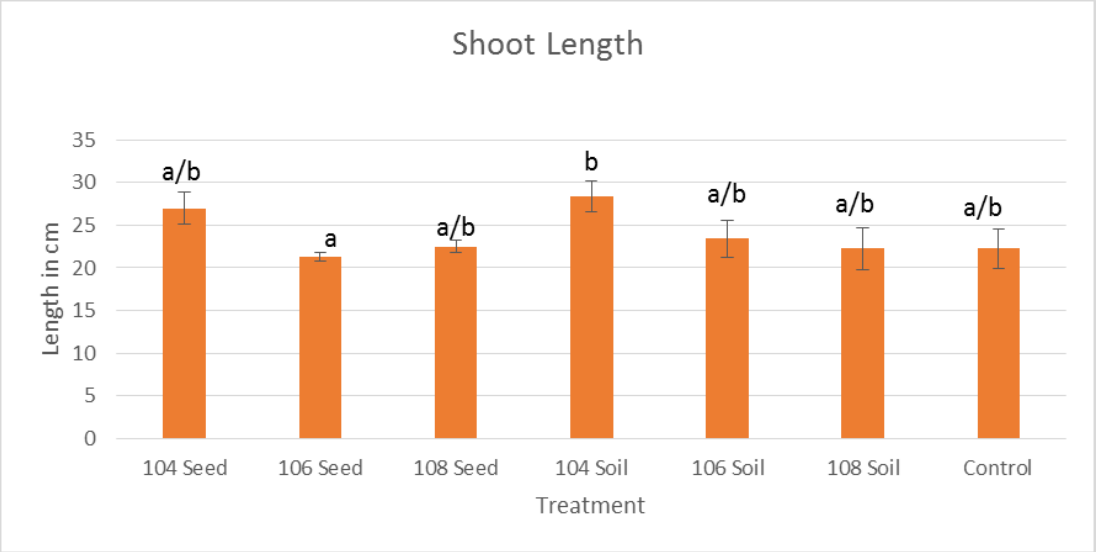


Fig. 4. Effect of *B. amyloliquefaciens* inoculation at different concentration on shoot length of rice plants 28 day after sowing. The columns represent the mean and standard error of shoot length recorded on 8 plants. Data represent the results of one experiment. Different letters indicate statistically significant differences (Duncan's multiple range test with $\alpha = 0.05$).

Concerning the analysis of root length (Fig. 5), there are no significant differences although, again, the best performing conditions are those where bacteria were inoculated at 10^4 cfu/mL in the substrate.

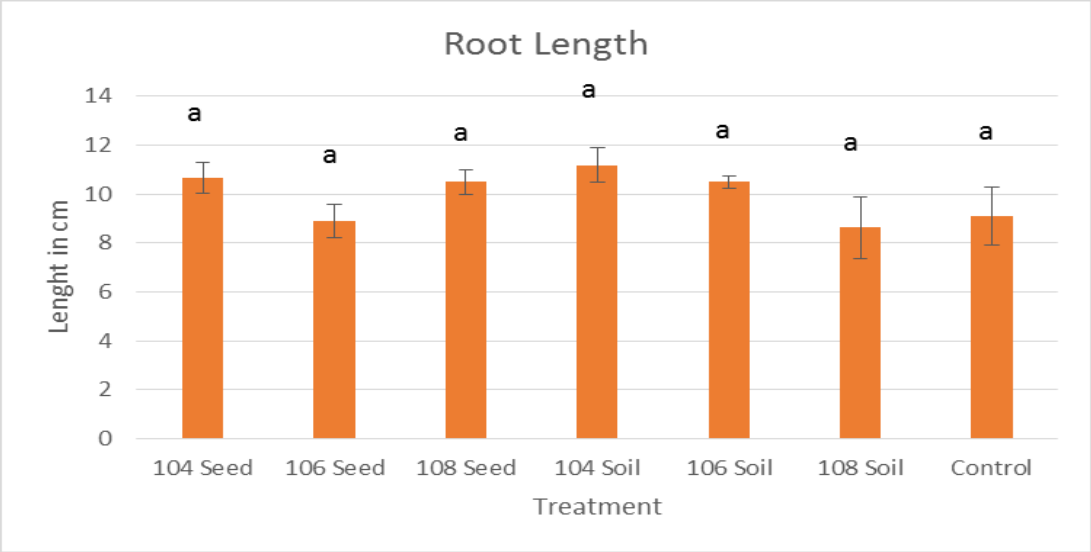


Fig. 5. Effect of *B. amyloliquefaciens* inoculation at different concentration on root length of rice plants 28 day after sowing. The columns represent the mean and standard error of root and shoot length recorded on 8 plants. Data represent the results of one experiment. Different letters indicate statistically significant differences (Duncan's multiple range test with $\alpha = 0.05$).

- *Shoot and root weight*

When the weight of shoots and roots were analysed, the same tendencies were observed so as the lowest concentrations of BaW1 stimulated the most the growth of plants. However, in this case some differences were statically significant. Bacteria promote the growth of shoots and root. In particular, shoot and root were heavier when bacteria were inoculated at 10^4 cfu/mL in soil, plants have heavier shoots compared with control, with significant differences.

Fig. 6. Effect of *B. amyloliquefaciens* at different concentration on shoot weight of rice plants 28 day after sowing. The columns represent the mean and standard error of root and shoot weight recorded on 8 plants. Data represent the results of one experiment. Different letters indicate statistically significant differences (Duncan's multiple range test with $\alpha = 0.05$).

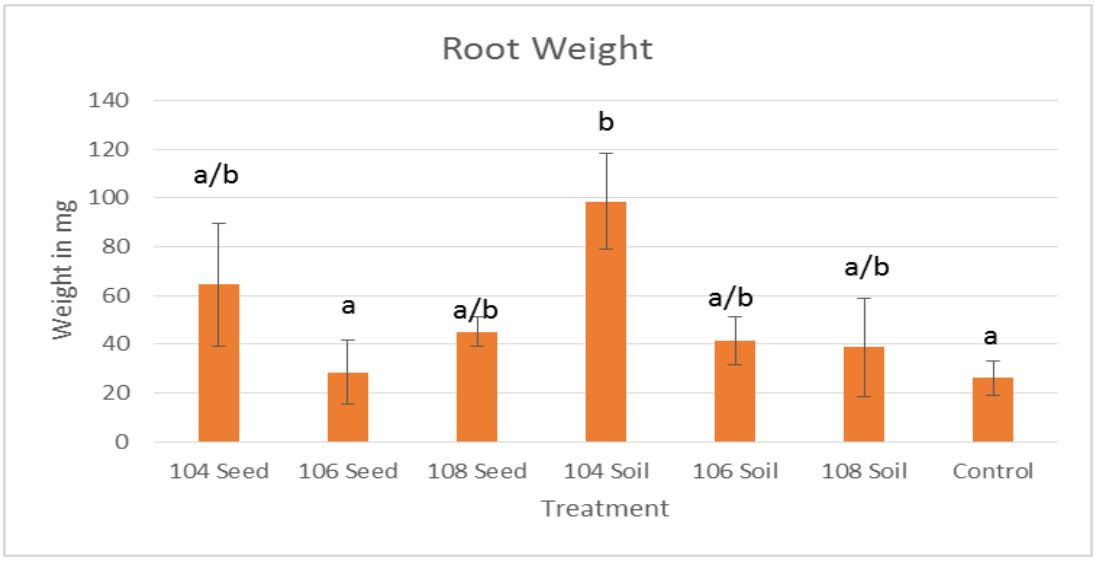


Fig. 7. Effect of *B. amyloliquefaciens* at different concentration on root weight of rice plants 28 day after sowing. The columns represent the mean and standard error of root and shoot weight recorded on 8 plants. Data represent the results of one experiment. Different letters indicate statistically significant differences (Duncan's multiple range test with $\alpha = 0.05$).

Two analyses were performed in order to verify if BaW1 colonized the surface of the root and to determine if they grow inside (endophytic) or at the surface of the root (rhizosphere) as explained above.

The sterilized roots didn't have any viable bacteria in any of the different concentrations and treatments as no growth was observed in the corresponding growth plates. On the other hand, non-sterilized roots from the second experiment, bacterial growth was observed in all concentrations and treatments. The control plate had also bacteria. Since the rhizosphere has plenty of PGPR, an identification should be carried out to identify what kind of bacteria had grown. Further experiments should be performed to stablish to which extent the bacterium is colonizing the root.

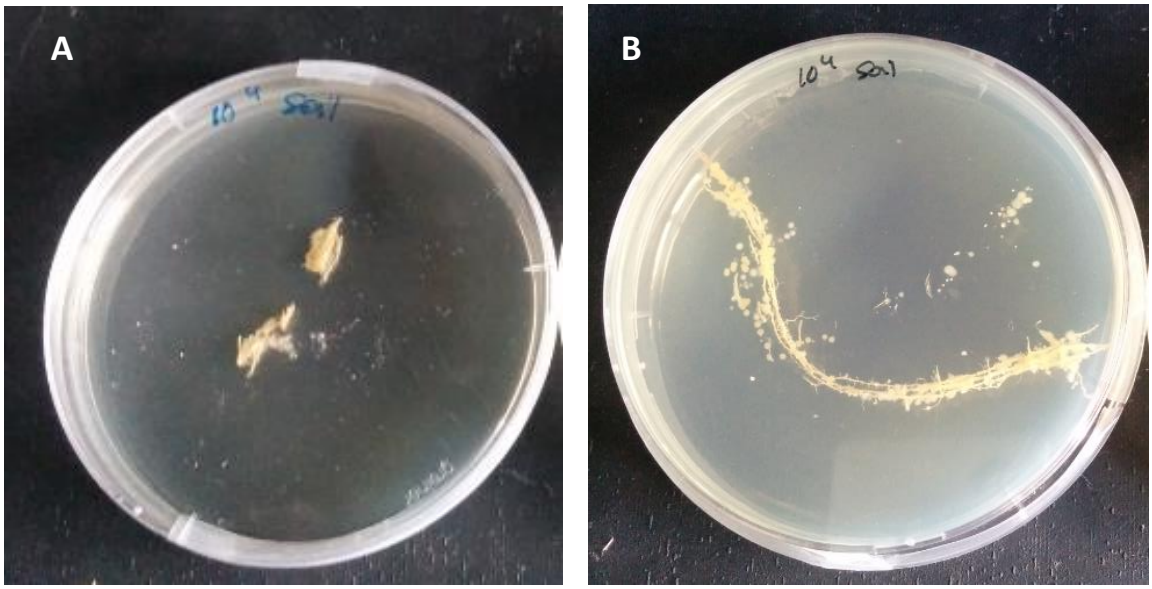


Fig. 8. Results of root colonisation test of 10^4 cfu/mL in soil treatment are shown in the image. **A**. Roots were surface sterilized. No bacterial growth can be observed. **B**. Roots were not surface sterilized. Around the roots, the rhizosphere, bacterial growth can be detected.

6.2.- Determination of *Ditylenchus angustus* infection in *Bacillus amyloliquefaciens* colonized rice plants

Based on the positive or neutral effects on rice growth, we decided to work further with BaW1-inoculation using the soil treatment.

The colonization of rice plants by *B. amyloliquefaciens* (soil treatment) resulted in significant reduction in the number of *Ditylenchus angustus* per plant (Fig. 9). It is observed that plants treated with 10^4 and 10^6 cfu ml^{-1} had a beneficial effect compared with control, reducing the infection almost 3 times. 10^8 cfu/mL didn't have a great effect, even though there was a statistically significant reduction in the infection compared with control. Bacteria BaW1 have a beneficial effect for the plant defences which is better at low bacterial concentrations.

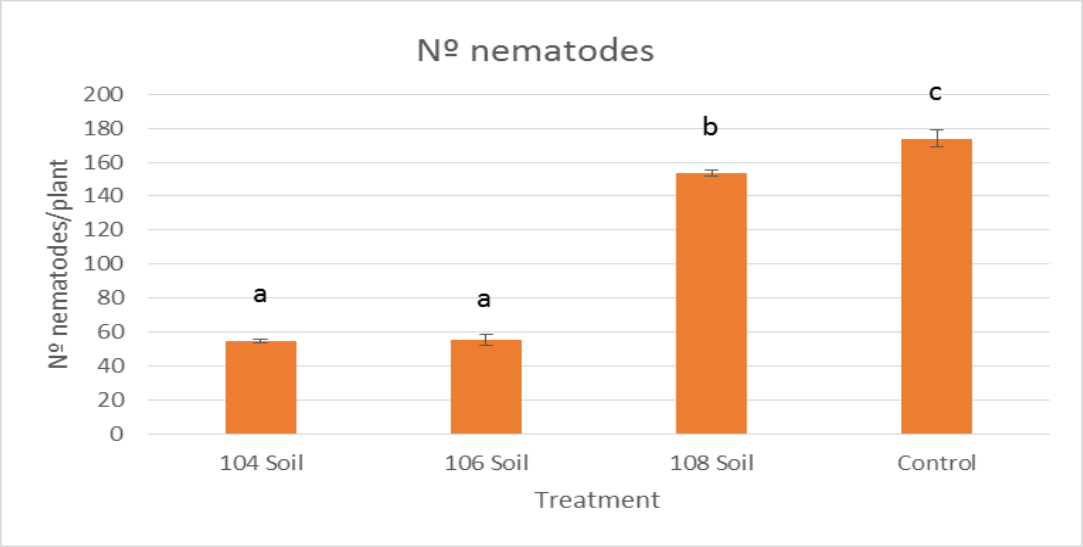


Fig. 9. Effect of *B. amyloliquefaciens* at different concentration on Ditylenchus angustus infection. At 15 days after sowing, nematodes were inoculated. After 15 days of inoculation, data was collected. The columns represent the mean and standard error recorded on 9 plants. Data represent the results of one experiment Different letters indicate statistically significant differences (Duncan's multiple range test with $\alpha = 0.05$).

6.3.- Establishment of relative gene expression by qPCR

Relative expression of different genes involved in plant defence pathways was measured by qPCR and compared with the expression of reference genes (Table 3). BaW1-inoculation was performed on day 12 after sowing. Nematode inoculation was done on the 13th day after sowing. Plant samples were collected on the 13th day after sowing and five days after nematode inoculation (DAI) so 18th after sowing. The experimental design is summarized in table 2.

Samples 13th day after sowing (no nematode infection)	**Samples 18th day after sowing**
Control (Plant untreated with bacteria)	Control (Plant untreated with bacteria or nematode)
Plant treated with 10^4 soil treatment, 1 day after inoculation	Plant untreated with bacteria + nematode infection (5 DAI)
	Plant treated with 10^4 soil treatment + nematode infection (5 DAI)

Table 2. Samples of the establishment of relative gene expression experiment. Samples were collected 13th days after sowing, and 1 day after 10^4 *B. amyloliquefaciens* inoculation. The same day, other plants were infected with *D. angustus*. After 5 days of infection, samples were collected. 2 biological replications were collected. Each biological replicate contains a pool of 4 plants.

The only experimental conditions which were analysed were the ones in which 10^4 cfu/mL of bacteria were inoculated in the soil, as they were the most beneficial conditions both for plant growth and plant defences compared with other treatments and concentrations.

Reference genes	Salicylic acid pathway (SA)	Jasmonate pathway (JA)	Ethylene pathway (ET)	Pathogenesis related genes (PR)
EXP	ICS1	AOS2	EIN2	PR10
EXP NARCAI	WRKY45	JAmyb	ACS1	

Table 3. Genes used for each pathway.

- *Effect of different conditions on gene expression*

Concerning the effect of the symbiosis between *B. amyloliquefaciens* and *O. sativa*, although the differential expression of all the genes is not significantly different from one, the results (Fig. 10) show a tendency were the two genes of the SA pathways would be downregulated. In the JA pathway on gene is upregulated while the other one is downregulated. In the ET pathway both genes are upregulated. Finally, PR10, which is a hub in the pathogenesis related pathways, tends to be upregulated.

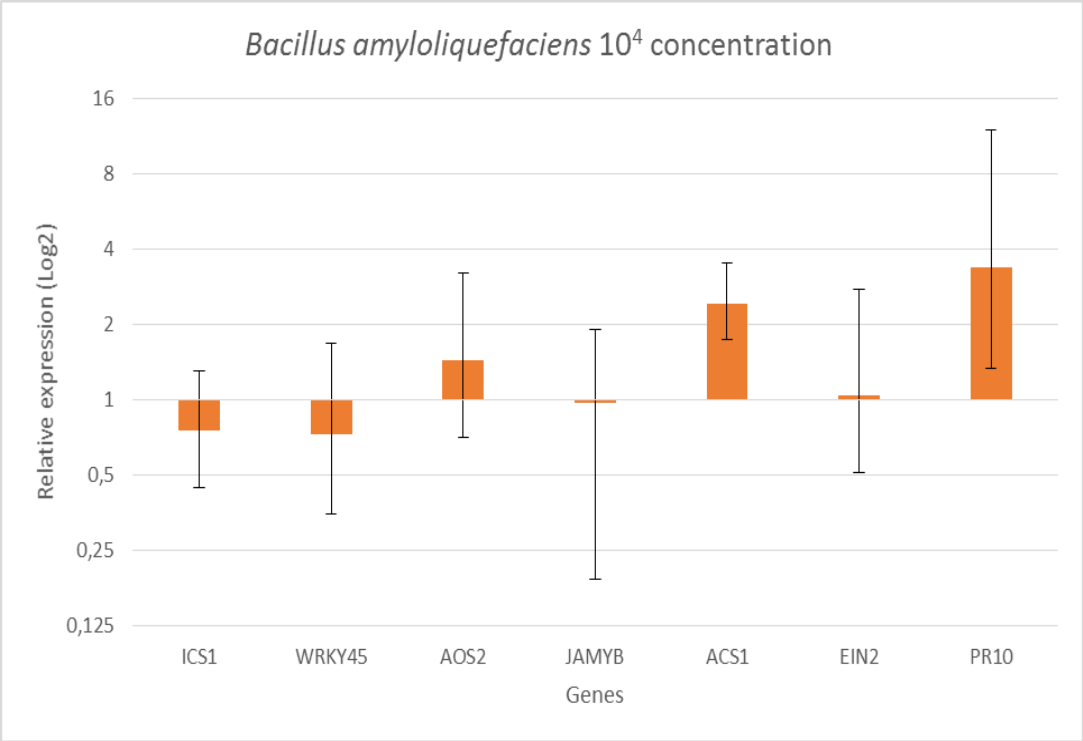


Fig. 10. qPCR data on some selected defence-related genes in shoot with *B. amyloliquefaciens* 10^4 cfu/mL inoculation. Bars represent the mean expression level and standard error from 2 biological replications and 3 technical replicates, each containing a pool of 4 plants. Data is shown as the relative expression level in log 2, where 1 represents the expression in non-inoculated plants. Bars above 1 indicate the gene is upregulated, the ones below 1 indicates the contrary, the gene is downregulated. Gene expression levels were normalized using 2 initial reference genes: EXP and EXP Narcai.

In the analysis of the effect of nematode challenge on gene expression, the results of the expression of the gene ACS1 were omitted because of a quality problem of the qPCR. For the rest of the genes there was no significant differential expression, thus, the results can only be described in terms of trends.

The expression trend of the genes AOS2, PR10 and WRKY45 was the same in all three experimental conditions, that is, in plants colonized by B. amyloliquefaciens, in plants infected with D. angustus, and in plants colonized by the bacterium and infected by the nematode. AOS2 and PR10 were upregulated while WRKY45 was downregulated. On the contrary, JAmyb, EIN2 and ICS1 have a trend change. JAmyb which tended to be downregulated in absence of the nematode, tends to be upregulated in presence of the nematode, in both cases, when the bacterium was present and when the bacterium was absent. EIN2 which tended to be upregulated in absence of the nematode, tended to be downregulated in presence of the nematode, in both cases, when the bacterium was present and when the bacterium was absent. Finally, the gene ICS1 tended to be downregulated both in plants colonized by the bacterium and in plants infected by the nematode, but it tended to be upregulated in plants infected with the nematode but colonized by the bacterium.

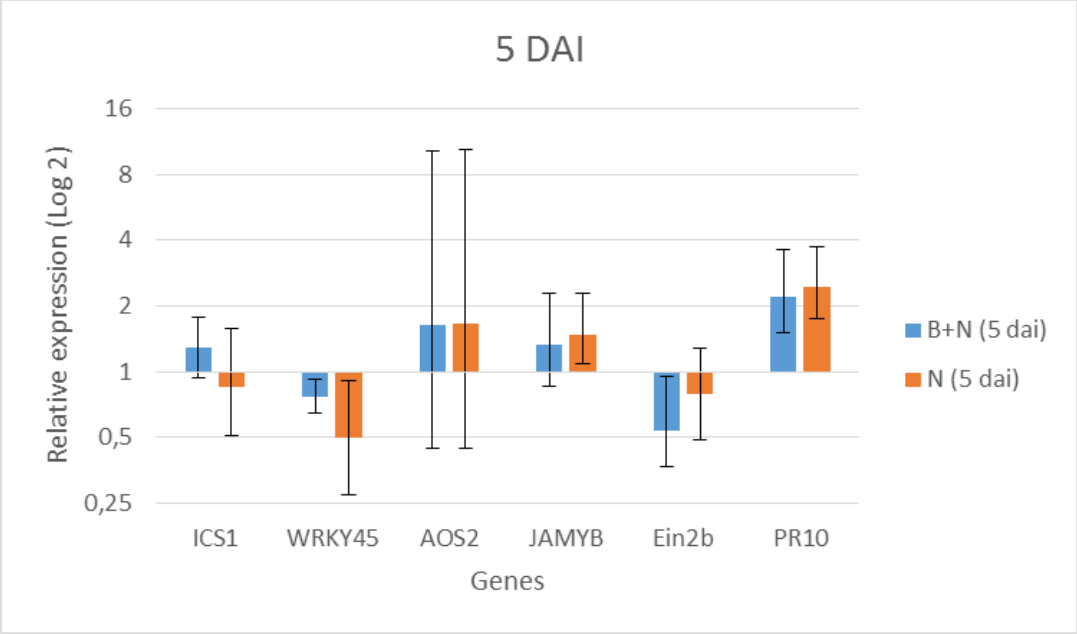


Fig. 11. qPCR data on some selected defence-related genes in shoot with *B. amyloliquefaciens* 10^4 cfu/mL inoculation and nematode. In orange, plants are only infected with *D. angustus*. In blue, plants were treated with *B. amyloliquefaciens* and infected with *D. angustus*. Bars represent de mean expression level and standard error from 2 biological replications and 3 technical replicates, each containing a pool of 4 plants. Data is shown as the relative expression level in log 2, where 1 represents the expression in the non-inoculated control plants. Bars above 1 indicate the gene is upregulated, the ones below 1 indicates the contrary, the gene is downregulated Gene expression levels were normalized using 2 initial reference genes: EXP and EXP Narcai.

7.- DISCUSSION

As described before, PGPR are bacteria that colonize plant roots and promote plant growth. They can affect the plant directly or indirectly. The direct effect is when PGPR provides the plant with a compound that is synthesized by the bacteria, exerting beneficial effects on the plant, such as hormones, or helping uptake certain nutrients from the soil (Beneduzi et al., 2012). They can fixate the atmospheric nitrogen, solubilize minerals such as phosphorus, produce siderophores than can solubilize and sequester iron, or even produce plant growth regulators, enhancing plant growth at various stages of development (Timmush, 2003).

As we were interested to know whether *Bacillus amyloliquefaciens* W1 is an endophytic bacteria, which colonize the interior of plant root or a PGPR we applied the surface sterilization technique (Lodewyckx et al., 2002). The results indicate that, when a surface sterilization is performed, no bacterial growth appears in the LB plate, meaning that bacteria are killed. Instead, there's growth of *Bacillus amyloliquefaciens* W1 when the surface is not sterilized which indicates that BaW1 might only be a PGPR but not an endosymbiont. However, more root colonization tests should be performed in order to conclude the nature of *B. amyloliquefaciens*.

The aim of seed germination experiments was to test whether our PGPR had a beneficial effect, like Shishido, 1996; Gutiérrez-Mañero et al., 2001; Ryu et al., 2003 described in their experiments with *Bacillus spp*. in various crops and weed species. However, Bozic et al., 2014 found *Bacillus spp.* to inhibit germination of *Cuscuta campestris* Yunck seeds. On the other hand, Ashrafuzzaman et al., 2009, in their seed germination tests, PGPR increased seed germination by 2.3 to 14.7 % over control. After seed treatment at different concentrations, 30 seeds were placed in a sterile wet paper petri dishes as described in materials and methods. Counting was done 5 days after incubation. The results, in figure 3, show that BaW1 doesn't have a beneficial effect on seed germination, on the contrary, at the highest concentrations some minor negative effects on seed germination was observed. Based on these observations it can be concluded that soil inoculation with 10^4 cfu/mL is the best inoculation method for BaW1 in rice plants.

In plant growth promoting experiments, the results also support the theory that *Bacillus amyloliquefaciens* is indeed a PGPR. As shown in figures 6, 7, soil treatment 10^4 is the bacterial concentration which helps the plant to grow bigger. In seed treatment, the results are similar, still, the best concentration is the lowest one, but not the best one in the overall picture. That could mean that the bacteria is preferentially colonizing the root, and not colonizing the seed tissues, or at least, not as efficiently.

In root length, there are no significant differences between treatments. However, roots might not be able to grow longer because of space limitations in the tubes used in the here-executed experiments (Ca. 15 cm long). Han & Lee, 2005, in their experiments with PGPR and growth of lettuce, discovered that the fresh and dry weight of lettuce under non-salinity

stress was increased by 13 % in the root length in comparison to the control treatment. Ashrafuzzaman et al., 2009 in their experiments evaluating the effiency of plant growth-promoting rhizobacteria for the enhancement of rice growth had an increase of root length in the plants inoculated with PGPR, root length ranged from 4.10 to 5.30 cm. Further experiments should be performed with longer tubes in order to verify if the space limitation is the cause of the observed similar length.

On the other hand, in root weight results, *Bacillus amyloliquefaciens* W1 is making a difference in 10^4 cfu/mL soil treatment, roots are heavier compared with other treatments. As mentioned before, bacteria might be colonizing the roots more efficiently when they are inoculated directly in the roots, and not in the seed. However, higher concentrations are not clearly beneficial for the plant, as seen in figures 6, 7.

Even though there are no significant differences in shoot length and between the BaW1-treatments and control, 10^4 cfu/mL soil treatment has the longest shoots. As a consequence, shoots are heavier. There is a relation between root weight and shoot weight. In 10^4 soil treatment, shoots are heavier, as well as roots. Since roots are heavier, the plant could get more nutrients from the rhizosphere, being able to survive and become stronger in comparison with the other plants. Roots grow through the soil, they come in direct contact with and intercept nutrients associated with soil particles that are displaced by roots. The nutrient uptake depends on the soil volume occupied by the roots (Adler & Cumming, n.d) . In fact, the lowest concentration in soil treatment had a 100 % of survivors, compared with other treatments and control, where all of them had only 75 % survivors (data not shown).

Many articles were published with similar results, PGPR promote plant growth. Huang et al., 2015 discovered that the inoculation of PGPR strains (*Bacillus spp*.) significantly promoted plant growth of corn and tomato when compared with control plants. Sunar et al, 2013, had similar results with Bacillus spp. Their results indicated that *B. altitudinis* was enhancing growth of three important legume crops, *Vigna radiate*, *Cicer arietinum*, and *Glycine max*.

As mentioned, PGPR can have a direct or indirect effect in plant growth. As described above, The direct effect is when PGPR provides the plant with a compound that is synthesized by the bacteria, exerting beneficial effects on the plant (Beneduzi et al., 2012). The indirect promotion occurs when PGPR prevent the deleterious effects of phytopathogenic organisms (Beneduzi et al., 2012). This indirect effect can occur via local antagonisms of soil born pathogens, or by induction of systemic resistance against pathogens throughout the entire plant (Timmush, 2003). Since *Ditylenchus angustus* attacks the plant shoots, no direct antagonism is to be expected here. BaW1-inoculation of the roots is capable to enhance plant resistance against the nematode at all concentrations. However, as shown in the previous analysis, the higher the concentration is, the more harmful BaW1 is for the plants and the less effect it has on the enhanced resistance against the nematode. In figure 9, the

number of nematodes decreases almost three times compared with control, when applying the lowest concentrations of BaW1. These results could confirm what Idriss et al., 2002, discovered, that *Bacillus amyloliquefaciens* strain FZB42 has de ability to suppress plant pathogens*. B. amyloliquefaciens*, as a PGPR, has the ability to produce antagonistic substances (Beneduzi et al., 2012) such as antibiotics, siderophores and a variety of enzymes (Timmush, 2003). Other investigations, such as, Burkett-Cadena et al., 2008, had similar results. Their results indicate that the suppression of nematodes (*Meloidogyne incognita*) and nematode damage was induced by various PGPR. These inoculants appear to suppress root-knot nematode via different mechanisms in tomatoes.

However, the bacteria might be activating ISR in the plant, upregulating the genes involved in JA/ET pathways, and, as a consequence, enhancing plant defence against plant pathogens. In order to verify what defence effect is triggered by *B. amyloliquefaciens* qPCR analysis was performed.

Plants can react to pathogen attacks thanks to a evolved complex mechanisms of signalling pathways that act both locally and systematically (Durner et al., 1997). Between this signalling pathways, SA, JA and ET are known to play key roles in plant defence. There are two systemically induced defence systems: ISR, where the defence is elicited by a non-pathogenic rhizobacteria and entails JA/ET pathways but not SA, and SAR, where the defence is induced by a pathogen or some chemicals, involving SA pathway and as a consequence the activation of PR-genes (Nahar et al., 2011). QPCR techniques were used in order to identify which pathways were activated or deactivated.

- *Effect of different conditions on gene expression*

As seen in graph 10, BaW1 has an effect on plant defence. JA and ET pathways are activated. AOS2 (JA-biosynthesis) and ACS1 (ET-biosynthesis) are upregulated meaning that there's an expression of these genes. However, JAmyb, another gene involved in Jasmonate pathway, is downregulated, and EIN2 is not differentially expressed. JA and its metabolites, are lipid-derived regulators that have key roles in plant defence and developmental processes. It is effective against necrotrophic pathogens and insects herbivores (David De Vleesschauwer, Gheysen, & Höfte, 2013). Furthermore, ET, is a major component in hormones released on a pathogen attack. It is believed that ET cooperates with JA in mounting immunity against necrotrophic pathogens (David De Vleesschauwer et al., 2013).

Regarding SA-related genes, WRKY45 and ICS1 are both downregulated, this pathway might not be activated by *B. amyloliquefaciens*, supporting the theory that JA/ET antagonizes SA-mediated biotroph resistance and act as independent pathways, they can work separately (David De Vleesschauwer et al., 2013). SA is a phenolic phytohormone which has a key role in thermogenesis, flowering, plant defence signalling, and systemic acquired resistance (Vlot, Dempsey, & Klessig, 2009). After the accumulation of SA in the plant, PR-genes are activated, leading a SAR defence (David De Vleesschauwer et al., 2013). SA accumulation, and as a result, SAR, can be triggered by exposing the plant to virulent, antiviruelnt, and non-pathogenic microbes, or artificially with chemicals (Vallad & Goodman, 2004).

PR genes, PR10, is upregulated. This is an expected result, since this gene is generally involved in all defence mechanisms activated to recognize any pathogen. In this case, since *Bacillus amyloliquefaciens* is not a pathogen, the plant might be considering the bacteria as a foreign system, activating then, the immune system defences. These responses are not limited to pathogen attack and can be induced by defence signalling molecules such as SA, JA and ET (Hwang, Lee, Yie, & Hwang, 2008).

Regarding the results represented in figure 11, where two samples are represented, the plant infected by the nematode alone and the plant infected by the nematode but with prior inoculation of the soil with *Bacillus amyloliquefaciens* at 10^4 cfu/mL.

Since *Ditylenchus angustus* is a pathogen, and as mentioned several times, SA is a primary defence pathway, so WRKY45 and ICS1 are expected to be upregulated. However, as represented in graph 11, both genes are downregulated, no expression is appreciated. This result might be indicating that the nematode has an effect inhibiting the SA pathway.

JA genes, AOS2 and JAmyb, are both activated. This might also indicate the nematode has an activation effect in the JA pathway. ET gene, EIN2 is downregulated, the nematode is having some inhibitory effect in this pathway as well. As expected, PR10 is upregulated.

In SA pathway, ICS1 is upregulated. When the plant is only inoculated with the nematode, the gene is downregulated. Furthermore, when the plant is BaW1-inoculated the gene is also downregulated. We believe that this explains why less nematodes are found. The SA-

pathway is activated, hence leading to resistance against the nematode. ISR is not strictly JA/ET, also SA can be involved, which might be the case here.

At the same time, WRKY45 is also downregulated. No clear conclusions can be made from this results, and more experiments should be carried out in order to understand the mechanism of this results.

In JA pathway, AOS2 is upregulated, with no differences with the plant only inoculated with the nematode, as well as JAmyb, which is also upregulated.

In ET pathway, EIN2 is also downregulated. Nematode might be having an inhibitory effect, and the bacteria concentration might not be strong enough to suppress the effects, since when only the plant is inoculated with bacteria, the gene is not differentially expressed.

At last, PR10 is upregulated, since is a gene activated when the plant realizes is being attacked by a foreign organism or a pathogen.

More experiments should be carried out in order to confirm these results, which are not clear. This is because of the large variation observed between the biological replicates, as can be seen in the error bars in the qPCR graphs.

8.- CONCLUSIONS AND FURTHER APPROACHES

The ability of some PGPR to suppress phytopathogens as well as promote growth in rice could be an important success for agriculture. No more chemicals would be used, making the process cheaper and healthier for the plants, the consumers and for the environment. Unfortunately, more experiments are needed to be performed to be finally able to understand the complex defence mechanisms of the plant. In this last part, conclusive statements of this research will be pointed out:

- *Bacillus amyloliquefaciens*, in soil treatment 10^4 cfu/mL is the most efficient treatment and concentration for the plant, increasing shoot and root weight with significant differences. In order to be able to determine if *B. amyloliquefaciens* has an effect on root length, larger Falcon tubes should be used. The shortness of Falcon tubes used in the growth analyses might be the reason why all roots have similar length.

- *Bacillus amyloliquefaciens* doesn't have a beneficial effect on seed germination. Furthermore, the higher the concentration is, the more harmful it is for the plant. However, we don't have statically evidence to confirm this phenomenon.

- *Bacillus amyloliquefaciens* might not be an endophytic bacteria but a PGPR, since after root surface sterilization, no bacterial growth was appreciated in LB plates. Bacteria were spotted in the plates without sterilization, but no identification was performed due to time limitations, so an identification should be done in order to accept that *B. amyloliquefaciens* is living in the rhizosphere.

- 10^4 cfu/mL and 10^6 cfu/mL soil treatments are the best concentrations for suppressing *Ditylenchus angustus* with significant difference between the other concentration (10^8 cfu/mL) and control.

- Results show that *Bacillus amyloliquefaciens* is activating genes involved in JA and ET pathways, but not SA pathway. JA/ET pathways might be working as antagonist with SA pathway. No clear results could be read from 5 DAI, more analysis should be carried out in order to understand the results collected.

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