University of São Paulo
"Luiz de Queiroz" College of Agriculture

Molecular mechanisms involved in the bacterial talking and maize growth promotion

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Dissertation presented to obtain the degree of Master in Science. Area: Genetics and Plant Breeding

Piracicaba
2018

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Bachelor of Biological Sciences

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Versão revisada de acordo com a resolução CoPGr 6018 de 2011

Advisor:
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Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Almeida, Jaqueline Raquel de

Molecular mechanisms involved in the bacterial talking and maize growth promotion / Jaqueline Raquel de Almeida. - -versão revisada de acordo com a resolução CoPGr 6018 de 2011. - - Piracicaba, 2018.

69 p.

Dissertação (Mestrado) - - USP / Escola Superior de Agricultura "Luiz de Queiroz".

1. *Bacillus* 2. Quorum quenching 3. PGPB 4. CRISPR-Cas9 5. *Azospirillumbrasilense* I. Título

Dedico este trabalho aos meus pais e ao Lucas
que sempre me deram apoio incondicional a todas as minhas escolhas.

AGRADECIMENTOS

Sou grata primeiramente a Deus, por todas as oportunidades de crescimento e aprendizado.

Sou grata aos meus pais e familiares pelo fundamental apoio e suporte durante esse período, permitindo que essa etapa fosse cumprida com alegria e reconhecimento.

Agradeço ao Lucas, pelo companheirismos, paciência, apoio e suporte durante essa caminhada, bem como todas as alegrias e lágrimas compartilhadas.

A querida amiga e orientadora Maria Carolina Quecine, pelo fundamental apoio, orientação e amizade, que auxiliaram muito em minha formação profissional e pessoal.

Ao profoessor João Lúcio pelo exemplo de dedicação, profissionalismo, e pelo apoio.

Sou grata a Renata, a Carolzinha, a Bruna, ao Everthon, a Isa e a Mari por fazerem parte dessa minha caminhada, pelos conhecimentos divididos, alegrias e risadas compartilhadas.

Agradeço a todo o pessoal do laboratório, Jessica, Helena, Maria Letícia, Renatinha, Bruno, Gustavo, Zezo, Joelma, Pedro, Daniel e Ines, pela convivência, conversas e auxílios.

Agradeço a Nathalia, pelo auxílio com as fotos de microscopia, ao Professor Roberto, pela disponibilização de sementes de milho e a Miriam pela ajuda.

Ao Professor Trevor Charles, da Universidade de Waterloo pelo acolhimento em seu laboratório e o auxílio no desenvolvimento da linhagem biosensora.

À FAPESP pelo suporte financeiro; À coordenação do programa de pós-graduação em Genética e Melhoramento de Plantas e a todos os professores do departamento, pelo auxilio a minha formação.

Muito obrigada!

“Se você não correr atrás do que quer, você nunca conseguirá.

Se você não perguntar, a resposta será sempre não.

Se você não for para frente, estará sempre no mesmo lugar.”

Nora Roberts

CONTENTS

RESUMO **7**

ABSTRACT **8**

1. INTRODUCTION **9**

REFERENCES **12**

2. DECIPHERING THE BENEFICIAL INTERACTION OF THE TROPICAL PLANT GROWTH-PROMOTING *BACILLUS* SP. RZ2MS9, *AZOSPIRILLUM BRASILENSE* AND MAIZE (*ZEA MAYS*) **15**

ABSTRACT 15
2.1. INTRODUCTION 15
2.2. MATERIAL AND METHODS 17
2.3. RESULTS 23
2.4. DISCUSSION 30

REFERENCES **34**

3. THE QUORUM QUENCHING SYSTEM IN A PLANT GROWTH-PROMOTING *BACILLUS* SP. RZ2MS9 ..... **41**

ABSTRACT 41
3.1. INRODUCTION 41
3.2. MATERIAL AND METHODS 43
3.3. RESULTS 52
3.4. DISCUSSION 59

REFERENCES **64**

RESUMO

Mecanismos moleculares envolvidos na comunicação bacteriana e na promoção de crescimento de milho

Concomitantemente ao aumento da produção agrícola, há o aumento do uso de fertilizantes minerais, que pode acarretar no desenvolvimento de diferentes problemas ambientais, além de causar a salinização dos solos. Uma possível alternativa para tentar reduzir a aplicação desses produtos é o uso de bactérias promotoras de crescimento de plantas (BPCPs), que podem ser usadas isoladamente ou em co-inoculação com outras bactérias, tornando-as uma alternativa ambientalmente e economicamente viável. Melhores resultados podem ser obtidos se a interação bactéria-bactéria e bactéria-planta for elucidada, permitindo que estratégias sejam desenvolvidas para otimizar essas interações. Em vista disso, a bactéria *Bacillus* sp. RZ2MS9, previamente descrita como uma potencial BPCP em milho e soja, foi marcada com GFP e monitorada durante a colonização de milho inoculada sozinha, bem como em co-inoculação com *Azospirillum brasilense* (Ab-v5::pWM1013). A interação dessas linhagens marcadas em milho, foi monitorada por microscopia de fluorescência (FM) e PCR quantitativo (qPCR), revelando um comportamento endofítico de *Bacillus* sp. RZ2MS9. Em plantas co-inoculadas, apesar da linhagem Ab-v5::pWM1013 não ter sido detectada por qPCR, a co-inoculação resultou no aumento do peso seco das raízes e da parte aérea, no volume e no diâmetro do sistema radicular, demonstrando que a inoculação com mais de uma linhagem bacteriana pode ser uma boa alternativa para o desenvolvimento de bio-fertilizantes. O *quorum sensing* (QS) é um importante sistema de comunicação célula-célula que permite que as bactérias reconheçam sua própria população e modulem sua expressão gênica. Este sistema também está envolvido na comunicação interespecífica, induindo outras espécies bacterianas e plantas. Co-evolutivamente, enzimas capazes de detectar e degradar essas moléculas evoluíram, dando origem ao chamado *quorum quenching* (QQ), sistema que evoluiu em algumas bactérias como uma vantagem competitiva para a colonização de nichos. O gene *aiiA*, foi um dos primeiros genes relacionados ao sistema QQ descrito no gênero *Bacillus*, gene este que foi anotado no genoma de RZ2MS9. Através da construção de uma nova linhagem biossensora de QQ, *Agrobacterium tumefaciens* At11006, e validada através da linhagem *A. tumefaciens* NTL4, a capacidade de RZ2MS9 de degradar moléculas de QS foi confirmada. O *knockout* do gene *aiiA* foi realizado utilizando o sistema CRISPR-Cas9, confirmando a função desse gene. Através dos resultados obtidos neste trabalho, a influência do sistema QQ de *Bacillus* sp. RZ2MS9 durante a colonização do milho, bem como a interação RZ2MS9 – *A. brasilense* – milho pode ser melhor investigada, abrindo a possibilidade de uma melhor compreensão do papel do sistema QQ na interação entre bactérias promotoras de crescimento e plantas.

Palavras-chave: *Bacillus sp. RZ2MS9; Quorum quenching; BPCPs; CRISPR-Cas9; Azospirilum brasilense*

ABSTRACT

Molecular mechanisms involved in the bacterial talking and maize growth promotion

With the increase of agricultural production, there is an improvement in the use of mineral fertilizers, which may cause different environmental problems, besides the soil salinization. A possible alternative for reducing the application of these products is the use of plant growth-promoting bacteria (PGPB), that can be used alone or in co-inoculation, resulting in an alternative environmentally and economically feasible. Better results can be obtained if the interaction among bacteria-bacteria and bacteria-plant be elucidated, and strategy developed to optimize these interactions. Thus, the plant growth-promoting *Bacillus* sp. RZ2MS9, previous described as a potential PGPB in maize and soybean, was GFP-tagged and monitored alone and co-inoculated with *Azospirillum brasilense* (Ab-v5::pWM1013) during maize colonization. The interaction of tagged strains in maize were monitored by fluorescent microscopy (FM) and quantitative PCR (qPCR), demonstrating an endophytic behavior of *Bacillus* sp. RZ2MS9. Although the non-detection of Ab-v5::pWM1013, the co-inoculation resulted in the best increase in root and shoot dried weight, root volume and in root diameter, showing that inoculation with more than one strain can be a good choice to development of bio-fertilizers. One important system to bacterial interaction is the quorum sensing (QS). The QS is an important cell-cell communication system that allows bacterial cells to recognize their own population and modulate their gene expression. This, system is also involved in the interspecific communication, induding other bacterial species and plants. In the other hand, enzymes able to detect and degrade these molecules evolved, the called quorum quenching (QQ) system, that has been evolved in some bacteria as competitive advantage for niches colonization. The *aiiA* gene, was one of the first gene related with the QQ in *Bacillus*. The *aiiA* was found in *Bacillus* sp. RZ2MS9 genome. Through construction of a new QQ biosensor, *Agrobacterium tumefaciens* At11006, and validated by *A. tumefaciens* NTL4, the ability of RZ2MS9 to degrade QS molecules was confirmed. The knockout of *aiiA* gene was performed using the CRISPR-Cas9 system, confirming this gene function. By these results, the influence of QQ system of *Bacillus* sp. RZ2MS9 during maize colonization and RZ2MS9 – *A. brasilense* - maize can be better investigated, opens the possibility to better understand the role of QQ system in the interaction among PGPB and plants.

Keywords: *Bacillus sp. RZ2MS9; Quorum quenching; PGPB; CRISPR-Cas9; Azospirilum brasilense*

1. INTRODUCTION

Along with the increasing in agricultural productivity, there is an improvement in the use of mineral fertilizers. Though, the excessive consumption of mineral fertilizers has roused environmental concerns, like eutrophication of fresh water bodies and proliferation of algal blooms in coastal waters (Ayoub, 1999) and economic impacts due to their high cost (Horrigan et al., 2002).

For agricultural production, nitrogen (N) is among the major mineral nutrients, interfering in several characteristics of plant growth and development (Cobucci, 1991). In Brazil, during the agricultural year of 2016, approximately 4.58 million tons of nitrogen were used, with more than 75% of this amount coming from imports (IPNI, 2016). Moreover, fertilizer consumption in 2016 showed a 250-fold increase compared to fertilizer used in 1995, 2.5 times higher than the increase around the world in the same period (IPNI, 2016).

In view of this scenario, efforts have been made to find microorganisms that have the capacity to maintain symbiotic relationships with crops and consequently help reduce the consumption of fertilizers, the called plant growth promoting bacteria (PGPB) (Parnell et al., 2016). One example is the PGPB *Azospirillum brasilense*, which has shown great potential for response in association with maize (Hungria et al., 2010). Among other possible promising strains, we highlight *Bacillus* sp. RZ2MS9, a PGPB isolated from the rhizosphere of guarana (*Paullinia cupana*) that expressively promoted maize and soybean growth under greenhouse conditions (Batista et al., 2018). The maize inoculation with RZ2MS9 increased the dry weight of root in 136.9% compared to the non-inoculated control. Furthermore, this PGPB *in vitro* assays was able to produce of indole acetic acid (IAA), siderophore, potential to biological nitrogen fixation and phosphate solubilization (Batista et al., 2018). These abilities were confirmed with the *Bacillus* sp. RZ2MS9 genome annotation (Batista et al., 2016). The authors have found 33 genes related to nitrogen fixation, 19 genes related to IAA production. It was found several genes for parts of the iron- and siderophore-uptake systems. In field experiments, maize inoculated with RZ2MS9 demonstrate an increase of 16 bags per hectare, compared with the control non-inoculated, using a lower nitrogen application than usual (Batista, 2017). These previous results demonstrate the potential of this strain to be used as bio-fertilizer.

Many studies have demonstrated that inoculation with more than one strain can improve the plant growth compared to single inoculation (Araújo et al., 2009). As instance, Santiago et al. (2017) demonstrated that the co-inoculation of potato with *Sphingomonas, Streptomyces, Methylibium* strains improved the potato growth. Similarly, Korir et al. (2017) using two PGPB (*Paenibacillus polymyxa* and *Bacillus megaterium*) under co-inoculation with rhizobia strains demonstrated the improvement of bean growth. In the present work, the co-inoculation of *Bacillus* sp. RZ2MS9 and *A. brasilense* Ab-v5, under

greenhouse conditions improved the dry weight, shoot height, root volume and diameter comparing with the non-inoculated control. These data corroborate with studies that have shown that co-inoculation with more than one microorganism can improve plant development (Hungria and Megías, 2013).

Few studies have related the co-inoculation and bacterial communication system, called quorum sensing (QS). This system appears to be implicated in the process of bacteria-bacteria and bacteria-plant interactions (Rosier et al., 2016). The QS system has been related with the regulation of various bacterial community's behaviors in the environment, including virulence factors production, motility, plasmid transfer, nodulation, antibiotic production and biofilm formation (Fuqua et al., 2001; Von Bodman et al., 2003; Whitehead et al., 2001). However, there are still incipient studies reporting the role of QS in plant growth promotion.

The need for an initial inoculum threshold level of PGPB to promote plant growth, strongly supports the idea that bacterial QS plays an important role in plant-PGPB interactions (Persello-Cartieaux et al., 2003). While the mechanisms of growth promotion appear to be universal, it is not known how the QS signaling by rhizobacteria allows the communication between PGPBs and their hosts, nor even how the PGPBs can modulate host gene expression (Rosenblueth and Martinez-Romero, 2006). Moreover, bacteria to promote the growth must be compatible with host plants, and it is possible colonizing plant tissues without being recognized as pathogens (Rosenblueth and Martinez-Romero, 2006).

In the other hand of QS system evolving, the quorum quenching system (QQ) arise to interfere in cell-cell communication by degrading the QS molecules. QQ system is important in niches in which different bacterial populations compete for resources, since the capacity for detection of QS molecules of another bacterial species is an adaptive advantage (Waters and Bassler, 2005). Many *Bacillus* species have QQ systems (Dong et al., 2002; Lee et al., 2002), these bacteria can secret an enzyme known as acyl homoserine lactonase (AiiA), encoded by the *aiiA* gene. This enzyme cleaves QS molecules from Gram-negative bacteria, the *N*-acyl homoserine lactone (AHL). Peculiarly, some Gram-negative bacteria also produce AHL lactonase. The *attM* gene, found *Agrobacterium tumefaciens* encodes an AiiA that controls AHL signal turnover in a growth-phase-dependent manner (Zhang et al., 2002).

The present work aimed to obtain the *Bacillus* sp. RZ2MS9-GFP tagged (RZ2MS9::PNKGFP), to understand your behavior during maize colonization, as well, study your interaction under co-inoculation with *A. brasilense* Ab-v5 during maize colonization. The co-inoculation of these strains improved the maize growth, demonstrating a synergistic interaction between them. The ability of this strain to colonize maize plants as an endophytic bacterium, as well the influence performed in the behavior of *A. brasilense* during maize co-colonization can be related with the production of AiiA enzyme, that will be better investigated (Chapter 1). The presence of AiiA enzyme in *Bacillus* sp. RZ2MS9,

was confirmed by plates assays. To confirm the AHL degradation ability of RZ2MS9, a new QQ biosensor strain, *Agrobacterium tumefaciens* At11006, was constructed. To confirm that ability of this strain in degrade AHL is due the presence of the *aiiA* gene, the influence of this gene was confirmed using the reverse genetics technique, obtaining defective mutants to AHL-lactonase production, using the double plasmid CRISPR-Cas9 system (Chapter 2).

Several studies demonstrated the beneficial interaction of PGPB and crops, although the use of bio-fertilizers still present a small fraction of the fertilizers currently used. In view of these, the better understanding of plant-bacteria interaction, including molecular traits, can help elucidate the problems of results inconsistency frequently reported, as well improve the results obtained through the use of PGPB.

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2. DECIPHERING THE BENEFICIAL INTERACTION OF THE TROPICAL PLANT GROWTH-PROMOTING *BACILLUS* SP. RZ2MS9, *AZOSPIRILLUM BRASILENSE* AND MAIZE (*ZEA MAYS*)

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ABSTRACT

The use of plant growth-promoting bacteria (PGPB) alone or in co-inoculation has been demonstrated as an environmental and economically feasible alternative compared to mineral fertilizers. In view of this, the plant growth promoter *Bacillus* sp. RZ2MS9 was GFP-tagged by electroporation and its interaction with the commercial bio-fertilizer *Azospirillum brasilense* (Ab-v5::pWM1013) and maize was investigated under greenhouse conditions. *In vitro* assays, RZ2MS9 GFP-tagged (RZ2MS9::pNKGFP6) demonstrated similar ability of RZ2MS9 wild-type to promote plant growth. Maize seeds were inoculated with RZ2MS9::pNKGFP6, and co-inoculated with RZ2MS9::pNKGFP6 and Ab-v5::pWM1013. The plants were evaluated 15 and 30 days after germination (DAG). The interaction of target strains in maize were monitored by fluorescent microscopy (FM) and quantitative PCR (qPCR). By FM, it was possible to observe that RZ2MS9::pNKGFP6 reached different maize tissues, colonizing the root system and aerial plant. But, RZ2MS9::pNKGFP6 density was similar in all maize tissues. Maize co-inoculated with Ab-v5::pWM1013 and RZ2MS9::pNKGFP6 also presented a decrease in the density levels of RZ2MS9::pNKGFP6 in the aerial plant tissues, at 15 day after germination, compared with maize only inoculated with RZ2MS9::pNKGFP6. However, the co-inoculation resulted in the best increase in root and shoot dried weight, root volume and in root diameter, showing that inoculation with more than one strain can be a good choice to development of bio-fertilizers.

Keywords: PGPB; gfp, Colonization; qPCR; Co-inoculation; Synergistic.

2.1. INTRODUCTION

Plant growth-promoting bacteria (PGPB) were first described by Kloepper and Schroth (1978) and represent symbiotic or free-living bacteria which are considered beneficial for plant growth and/or plant development. The main genera of PGPB are: *Acinetobacter*, *Agrobacterium*, *Arthobacter*, *Azospirillum*, *Azotobacter*, *Bacillus, Bradyrhizobium*, *Burkholderia*, *Frankia*, *Pseudomonas, Rhizobium*,

Serratia and *Thiobacillus* (Glick 1995, Vessey 2003). Among them, *Bacillus* is one of the better studied PGPB groups (Beneduzi et al., 2012).

PGPB include soil and rhizospheric free-living cells, symbiotic cells and endophytes that can colonize some or a portion of a plant's interior tissues (Glick, 2012). They are able to perform different biological function, resulting in a reduction of the use of antibiotics, herbicides, pesticides and other artificial supplements (Adesemoye et al., 2009; Ahemad and Khan, 2009; Bhattacharyya and Jha, 2012; Lucy et al., 2004). PGPB can be divided into two classes: biocontrol agents which indirectly benefit the plant; and plant growth promoters, that show the ability to affect directly the plant metabolism (Bashan and Holguin, 1997).

Concerning the PGPB multi-traits: they are able to improve the plant growth through many mechanisms (Glick, 2012): phytohormones production (Patten and Glick, 1996), biological nitrogen fixation (Hurek et al., 2002), phosphorus solubilization (Rodriguez et al., 2004; Vikram and Hamzehzarghani, 2008), upgrading the nutrient absorption (Kraiser et al., 2011); antimicrobial metabolites production (Raaijmakers et al., 2002) and by competition for niches and nutrients (Paterson et al., 2016). PGPB may also trigger the induction of plant systemic resistance (Bakker et al., 2007). It is worth highlighting that a single bacterium can perform concomitantly more than one of those mechanisms (Ahmad et al., 2008). Although, several products formulated with PGPR are commercially available as biofertilizers and biocontrol agents (Gohel et al., 2006; Jha and Saraf, 2015; Sethi et al., 2014). However, PGPR inoculated crops represent only a small fraction of current worldwide agricultural practice (Glick, 2012). Therefore, the knowledge concerning the PGPB-plant interaction and the basic traits involved in the plant growth promotion must be improved (Goswami et al., 2016).

It is known that the combination of many microorganisms in soil give rise to a stable ecosystem through the synergistic interactions of compatible microbes, resulting in increased plant productivity (Pandey et al., 2012). The co-inoculation, frequently, increase growth and in some cases yield, compared to single inoculation, due to the provide plant balanced nutrition, improving absorption of nitrogen, phosphorus, and other mineral nutrients (Araújo et al., 2009). In rice, the co-inoculation with *Azospirillum* spp. demonstrated better results in contrasting of the single inoculation (Amutha et al., 2009). Similar results were observed in banana co-inoculating rhizobacteria (*Azospirillum brasilense* Sp7, *Bacillus sphaericus* UPMB10 and *Microbacterium oxydens* UPMB11) and agrobacteria (*Agrobacterium rhizogenes* strains, AR9402 and A4) (Mahmood et al., 2010). *Sphingomonas*, *Streptomyces*, *Methylibium* strains in consortium also improved the potato growth (Santiago et al., 2017). The co-inoculation of PGPR and *Rhizobia* improved the bean growth (Korir et al., 2017).

Brazil is the third biggest maize producer worldwide, being expected a production of 96 million tons in 2018 (USDA, 2018). The use of *A. brasilense* strains Ab-V5 and Ab-V6 on maize (*Z. mays* L.) and wheat (*T. aestivum* L.) crops in Brazil has grown exponentially (Hungria et al., 2010). *A. brasilense* may

promote the plant growth and have been described eliciting plant resistance to biotic and abiotic stresses (Fukami et al., 2017). Another promising PGPB to be used in maize crop production is *Bacillus* sp. strain RZ2MS9, bacterium isolated in Brazil from guarana's rhizosphere. The inoculation of this strain in maize seeds, under greenhouse conditions, demonstrated an increase of 235.5 % in the dry weight of maize shoots, and 136.9 % in maize root dry weight, compared to the non-inoculated control at 60 days after seeding (Batista et al., 2018).

Thus, this work aimed the better understanding the interaction between *Bacillus* sp. RZ2MS9 – maize as well as the synergistic interaction between *Bacillus* sp. RZ2MS9 and *A. brasilense* during maize colonization by monitoring of RZ2MS9 GFP tagged, and the plant responses by co-inoculation of RZ2MS9 and *A. brasilense* Ab-v5., previously tagged with dsRed.

2.2. MATERIAL AND METHODS

2.2.1. Bacterial strains and growth conditions

Bacillus sp. RZ2MS9, a plant growth promoter isolated from guarana (*Paullinia cupuna*) (Batista et al., 2018), and its derivate tagged strains, as well as *Escherichia coli* derivatives were routinely growth on Luria-Bertani (LB) medium (Sambrook et al., 1989) using appropriated antibiotic at 28°C and 37°C, respectively. The tagged strain *A. brasilense* Ab-v5::pWM1013 (Tschoeke, 2016) were grown on DYGS medium (Rodriguez et al., 2004) supplemented with 50 $\mu g.ml^{-1}$ kanamycin sulfate at 28 °C. All the strains (Table 1) were stored in 20 % glycerol at -80°C. The plasmids were propagated and isolated from *E. coli* DH5α *pir* or Top10 and purified with a plasmid miniprep kit (Qiagen) according to the manufacturer's recommendations.

Table 1. Plasmids and strains used in this work

Plasmids	Description	Reference
pNKGFP	Integrative plasmid: 5.6 kb; ORI R6K; mini-Tn10; *gfp* gene and Km^R	Ferreira et al., 2008
pBAV1K-T5*gfp*	Replicative plasmid: 3.6 kb; ORI Orf D; repA; *gfp* gene and Km^R	Bryksin and Matsumura, 2010
pCM88	Replicative plasmid: 8.0 kb; ORI V, ORI T, colE1, *gfp* gene and $Tetr^R$	Marx and Lidstrom, 2001
Strains	**Description**	**Reference**
E. coli DH5α	*pir recA1 endA1 hsdR1 relA1* ::*pir*	Kolter et al., 1978
Bacillus sp. RZ2MS9	PGPB from guarana (*Paullinia cupana)*	Batista et al., 2018
Bacillus sp RZ2MS9::pNKGFP	RZ2MS9 tagged with *gfp* gene and Km^R	This work
A. brasilense Ab-v5::pWM1013	Ab-v5 tagged with dsRed and Km^R	Tschoeke, 2016

2.2.2. Transformation of *Bacillus* sp. RZ2MS9

RZ2MS9 transformants were obtained by electroporation according to the protocol described by Schurter et al. (1989) with modifications. Briefly, one single colony of RZ2MS9 was inoculated in 10 ml of LB with 0.1 % of glycine and incubate overnight at 28°C on shaker at 150 rpm. The culture was diluted 100-fold (optical density - OD_{550nm}: 0.01) in 100 ml of LB with glycine 0.1 % and incubated until an OD_{550nm}: 0.2. The cell was obtained by centrifugation and resuspended in 1/40 volume ice-cold electroporation buffer (400 mM sucrose, 1 mM $MgCl_2$, 7 mM phosphate buffer, pH 6.0), twice. The cells were resuspended into 2.5 ml of electroporation buffer and 800 µl aliquots of the ice-cold cells were distributed into precooled Genepulser cuvettes. 30 ng of plasmid DNA was added and kept for 10 min at 4°C. Electroporation was performed using: 25 µF capacitor and 1.3 kV, in Bio-Rad Genepulser. The cuvettes were maintained another 10 min at 4°C, diluted into 1.2 ml LB and incubated for 2 h at 28°C on rotatory shaker at 150 rpm. The transformants were detected in LB agar plates with the appropriate antibiotic after 24 hours of growth at 28°C. The replicative plasmids pBAV1K-T5gfp and pCM88 were used to optimize the RZ2MS9 transformation. The integrative plasmid, pNKGFP, was chosen to perform the monitoring of RZ2MS9 in maize, by fluorescent microscopy. Some RZ2MS9::pNKGFP transformants were randomly selected and grown in LB broth medium containing 50 $\mu g.ml^{-1}$ kanamycin sulfate and preserved in 20 % glycerol at -80° C.

2.2.3. Development of stable GFP-tagged RZ2MS9

2.2.3.1. Molecular confirmation of transformation

One single colony of RZ2MS9 wild-type, RZ2MS9::pNKGFP1, RZ2MS9::pNKGFP2 and RZ2MS9::pNKGFP6 transformants were inoculated into 5 ml of LB broth medium supplemented with 50 $\mu g.ml^{-1}$ kanamycin and maintained overnight at 28°C and 150 rpm. Bacterial cells were collected by centrifugation and bacterial genomic DNA was extracted using the DNeasy® blood and tissue kit (Qiagen) following the manufacturer's recommendations. DNA integrity was verified on 1 % agarose gel, stained with 0.5x SYBR® Green (Invitrogen®) and quantified in NanoDrop™ spectrophotometer (NanoDrop Technologies).

To confirm the transformation of the RZ2MS9 with pNKGFP plasmid, the amplification of an internal region of the plasmid was performed using the primers: PPNKF (5 'CCTTCATTACAGAAACGGC 3') and PPNKRII (5 'GGTGATGCGTGATCTGATCC 3') (Quecine et al., 2012). pNKGFP was used as positive control. RZ2MS9 wild-type DNA and DNA-free water were used as negative controls. The reaction was performed with 0.75 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTP (10 µM), 2.5 µl of 10X Buffer, 0.5 µl of each primer (10 µM), 0.3 µl of Taq DNA polymerase, 19 µl of water, yielding a final volume of 25 µl reaction. The PCR program consisted of an initial denaturation step at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 30 s. The final step was a 10 min extension at 72°C. The amplified products were separated by electrophoresis with a 1 % agarose gel and stained with 0.5x SYBR® Green (Invitrogen®).

2.2.3.2. Growth curve

To verify if the insertion of the pNKGFP modified the growth of the transformants, RZ2MS9 wild-type, RZ2MS9::pNKGFP1, RZ2MS9::pNKGFP2 and RZ2MS9::pNKGFP6 were grown in 50 ml of LB medium. RZ2MS9::pNKGFP6 was also grown in LB medium supplemented with 50 $\mu g.ml^{-1}$ of kanamycin. Bacterial liquid cultures, four replicates for each strain were diluted and standardized (OD_{600nm}: 0.2) in 50 mL of LB medium being incubated at 28°C in a rotatory shaker (150 rpm). The OD_{600nm} was measured each two hours intervals, for 48 hours to monitor bacterial growth (spectrum).

2.2.3.3. *In vitro* plasmid stability

Three transformants containing the plasmid (RZ2MS9::pNKGFP1, RZ2MS9::pNKGFP2 and RZ2MS9::pNKGFP6) were first grown in LB broth medium supplemented with kanamycin for 12 hours

and then diluted to OD_{600nm}: 0.2 in 50 ml of LB broth medium without antibiotic. The cultures were grown for approximately sixty generations (2h by generation), and cells (10^3 CFU.ml^{-1}) from the 6th generation were grown for another 6th generation (five times), in a total of 120 h hours. A fraction of cultures at late log phase was used for a dilution series, 10^{-10}, 10^{-11} and 10^{-12}. Aliquots of 100 µl of each dilution were plated on LB medium without kanamycin and then incubated at 28°C for 24 hours. A hundred random single colonies were inoculated using toothpick on LB agar medium supplemented with 50 µg.ml^{-1} of kanamycin and incubated at 28°C for 24 hours. Colonies were counted and the percentage of clones carrying the plasmid was calculated. All evaluation was performed in triplicate.

2.2.3.4. Seed germination test

The influence of *Bacillus* sp. RZ2MS9 wild-type and RZ2MS9::pNKGFP6 in the initial development of maize seedlings was performed using Dupont Pioneer® P4285H seeds. The maize seeds were washed twice in distilled water and then immersed in bacterial solution (OD_{600}: 0.12), being kept under stirring for 30 min.

After bacterization, the seeds were placed into Petri dishes, between seed germination paper towel moistened and incubated in the dark at 28°C for 7 days. The root growth was assessed by scanner. The images were captured at 400 dpi resolution with the Epson® Expression 11000XL scanner and analyzed using the software WinRHIZO Arabidopsis (Regent Instruments Inc., Quebec, Canada). The parameters: superficial area, root volume, root length, lateral root, axial root and diameter were statistically analyzed by Tukey and T test. It was used 30 seeds per plate and four replicates per treatment.

2.2.4. Maize co-colonization by RZ2MS9 and Ab-v5 tagged strains

The interaction between *Bacillus* sp. RZ2MS9 - maize, as well the interaction among RZ2MS9 – Ab-v5 - maize were carried out as completely randomized assay under greenhouse conditions located in "Luiz de Queiroz" College of Agriculture, University of São Paulo, Piracicaba – SP, Brazil (22° 42′ 30″ S and 47° 38′ 30″ W), in May 2016. The treatments were: T1 (non-inoculated control), T2 (inoculation of RZ2MS9::pNKGFP6) and T3 (co-inoculation of RZ2MS9::pNKGFP6 and Ab-v5::pWM1013). Maize seeds, hybrid P4285H DuPont® Pioneer®, were kindly provided by Prof. Dr. Roberto Fritsche-Neto, Department of Genetics at ESALQ/USP.

Seed inoculation were performed according to Batista et al. (2018). The seeds were immersed in the bacterial solution containing 10^8 CFU.ml^{-1}. Previously, culture cells were obtained growing each

bacteria in 100 ml of LB and DYGS broth media supplemented with Kanamycin 50 µg.ml^{-1} to RZ2MS9::pNKGFP6 and Ab-v5::pWM1013 respectively. The strains grew at 28°C in shaker (150 rpm) until achieve the concentration of 10^8 CFU.ml^{-1} (OD_{600nm}: 0.12). The seeds were immersed and maintained in the bacterial solution for 30 min and then sown. For control, seeds were immersed and maintained in bacterial medium diluted 10 times for 30 min and then sown.

Maize seeds were planted in pots with 1.6 kg of the thick, branny substrate Bioplant® (http://www.bioplant.com.br/), which is composed of peat, correctives, vermiculite, charcoal and pine bark (from Bioplant Agrícola Ltda.). Four seeds were sown per pot and kept under greenhouse conditions under daily irrigation with 230 ml of water. On the 8th day after germination (DAG), thinning was performed, living two plants per pot until the 15th DAG, when one plant of each pot was removed for the first evaluation. The second evaluation was performed at 30th DAG with the remaining plants.

The root systems and aerial parts were collected 15 DAG and 30 DAG and the fresh tissues were cut and immediately observed using the epifluorescence microscope Axiophot II (Zeiss, Germany) with the following filter sets (excitation/emission): 365/397 nm for blue, 450/515 nm for green and 546/590 nm for red. Images were digitalized through a PCO CCD camera using ISIS Metasystems software (Metasystems, Germany).

For quantification by qPCR, immediately after each evaluation (15th and 30th DAG), four plants of each treatment were stored at - 80° C for later DNA extraction.

2.2.5. Quantitative PCR

Four biological replicates of each treatment, of each sampling period, were used for bacterial quantification. The total DNA was extracted using the DNeasy® Plant Mini Kit (Qiagen) following the manufacturer's instructions. DNA integrity analysis was performed using 1 % agarose gel electrophoresis, stained with 0.5x SYBR® Green (Invitrogen®) and observed under UV light. DNA quantification was performed using the NanoDrop ™ spectrophotometer (NanoDrop Technologies).

For RZ2MS9::pNKGFP quantification, the primers PPNKF and PPNKRII (Quecine et al., 2012), that amplify an internal region of pNKGFP plasmid. And, for *A. brasilense* Avb-5::pWM1013, the quantification was performed with the primers AzoR2.1F (CGCCACCATGCGATCAA), AzoR2.1R (GCATGCCCAGTACTGCAAGTC) (Stets et al., 2015), specific for *A. brasilense* detection.

The qPCR analysis was performed in a 12 µL final volume, which contained 6.25 µL of the Platinum® qPCR superMix-UDG (Invitrogen), 0.25 µL of each primer, PPNKF and PPNKRII (10 µM) and 0.25 µL of Bovine Serum Albumin (BSA). Aliquots of the master mix (7 µL) were dispensed in the wells and 5 ng of DNA was added as a PCR template. The qPCR cycles consisted of denaturation step at 95°C

for 2 minutes, 40 cycles at 95°C for 30 seconds and a final step at 62°C for 15 seconds. The plasmid fragment quantification was performed using an iCycler iQ real-time PCR instrument (BioRad Laboratories Inc.). Three biological replicates were used in duplicate, and a standard curve was obtained for each run using a known copy number (10^4 to 10^{10}) of the linearized plasmid pNKGFP. The number of colony forming units (CFU) within the tissue per nanogram of total plant DNA was estimated from standard curves, that were generated by dilutions of PCR products with primers PPNKF and PPNKRII using the plasmid pNKGFP as template. The product was quantified in NanoDrop™ spectrophotometer and the number of copies of the gene per µL of product was calculated (Staroscik, 2004).

For *A. brasilense* Ab-v5::pWM1013, the qPCR was performed in a total reaction volume of 25 µl containing 12.5 µl Platinum® qPCR superMix-UDG (Invitrogen), 6.25 µl of a primer AzoR2.1F and AzoR2.1R mix (final concentration, 1 µmol), and 6.25 µl of 2.5 ng/µl diluted template DNA. The cycling program included a 10 min incubation at 95°C, 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds followed by 72°C for 30 seconds, and an additional incubation at 72°C for 10 minutes. The bacterial quantification was performed using an iCycler iQ real-time PCR instrument (BioRad Laboratories Inc.). Three biological replicates in duplicate were used.

The standard curves used for the quantification of *A. brasilense* Ab-v5::pWM1013 in maize were constructed as described previously (Stets et al., 2015). Maize plants were grown under greenhouse condition, and roots were collected and crushed in liquid nitrogen using a mortar and pestle. A volume of 100 µl of an *A. brasilense* Ab-v5::pWM1013 culture (dilution range 10^2 to 10^9 CFU) was added to 100 mg of crushed roots, and the components were mixed and incubated for 1 h at room temperature. The whole mixture was used for DNA extraction with DNeasy® blood and tissue kit (Qiagen) following the manufacturer's recommendations. qPCR was performed as described above. The standard curve was generated by plotting the CT value versus the number of CFU added to each tube. No bacteria were added to the negative control.

2.2.6. Plant growth promotion analysis

Six biological replicates were used to evaluate the influence of RZ2MS9::pNKGFP6 and the co-inoculation of RZ2MS9::pNKGFP6 and Ab-v5::pWM1013 in maize growth. The evaluated parameters were: shoot height, diameter, fresh and dry shoot weight and root dry weight. The root system of each sample was placed in 70 % alcohol solution until be evaluated through a scanner. The roots were scattered in a clear layer of water in a tray (30 cm by 20 cm), and the image were captured at 400 dpi (dots per inch) with an Epson® Expression 11000XL professional scanner system. The obtained images were analyzed using the software WinRHIZO Arabidopsis (Regent Instruments Inc., Quebec, Canada),

obtaining values of morphological characteristics of the root system as: diameter, root length, axial root length, lateral root length, surface area and root volume. Ten diameter classes were provided by the software and simplified in only two, axial and lateral root length (Trachsel et al., 2009). Thus, for the lateral root length, the fragments with a diameter of less than or equal to 0.5 mm were considered, and for the axial roots, the fragments with a diameter greater than 0.5 mm, were used.

2.2.7. Statistics analyzes

All data were statistically analyzed by variance analysis with the software R (RCore Team 2017), considering the experimental design as completely randomized for the bioassays, and using the Tukey and T test, the means were considered significantly different when p-value was <0.5 and <0.1, respectively.

2.3. RESULTS

2.3.1. Construction of RZ2MS9::pNKGFP stable tagged strain

The transformation protocol for RZ2MS9 was efficient using replicative and integrative plasmids. The efficiency of transformation using pNKGFP was higher ($8x10^3$ transformants per µg of plasmid DNA) than using pBAV1K and pCM88 ($4.7x10^3$ and $4.2x10^3$ transformants per µg of plasmid, respectively).

The transformation of RZ2MS9 with pNKGFP plasmid was confirmed by PCR, performed with the specifics primers for the plasmid, PPNKF and PPNKRII, showing an amplicon of 362 pb (Figure 1). It was not observed when used wild-type DNA as template.

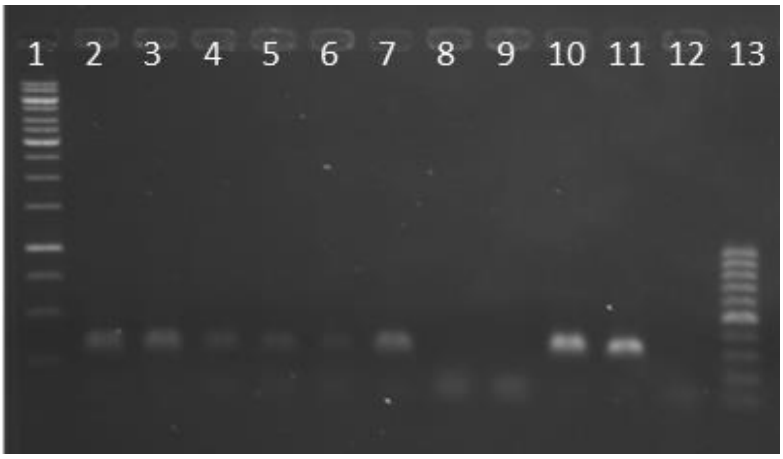

Figure 1. Molecular confirmation of RZ2MS9::pNKGFP transformants by PCR reaction using the primers PPNKF (5 'CCTTCATTACAGAAACGGC 3') and PPNKRII (5 'GGTGATGCGTGATCTGATCC 3') (Quecine et al., 2012). Lanes: **(1)** DNA ladder 1kb (Fermentas®); **(2)** and **(3)** RZ2MS9::pNKGFP1; **(4)** and **(5)** RZ2MS9::pNKGFP2; **(6)** and **(7)** RZ2MS9::pNKGFP6; **(8)** and **(9)** RZ2MS9 WT (negative control); **(10)** and **(11)** pNKGFP plasmid (positive control); **(12)** DNA-free water (blank control); **(13)** DNA ladder 100pb (Fermentas®).

The insertion of the plasmid pNKGFP had no effect on bacterial growth, independently of antibiotic addittion. All bacterial strains showed the starting of the log phase approximately at 2.5 hours after inoculation (h.a.i.) and reached the stationary phase 11 h.a.i. (Figure 2). As expected, the plasmid in the genome of *Bacillus* sp. RZ2MS9::pNKGFP6 remained stable 120 hours growing without antibiotic.

The evaluation of RZ2MS9::pNKGFP6 strain on maize seeds germination demonstrated that the transformation did not influence its ability to induce the plant growth promotion (Figure 3).There was no statistical difference between the treatment inoculated with RZ2MS9::pNKGFP6 and RZ2MS9 wild-type for all evaluated parameters. However, the bacterial treatments differed significantly to the non-inoculated control, according to Tukey's test, in all evaluated parameters (Figure 3).

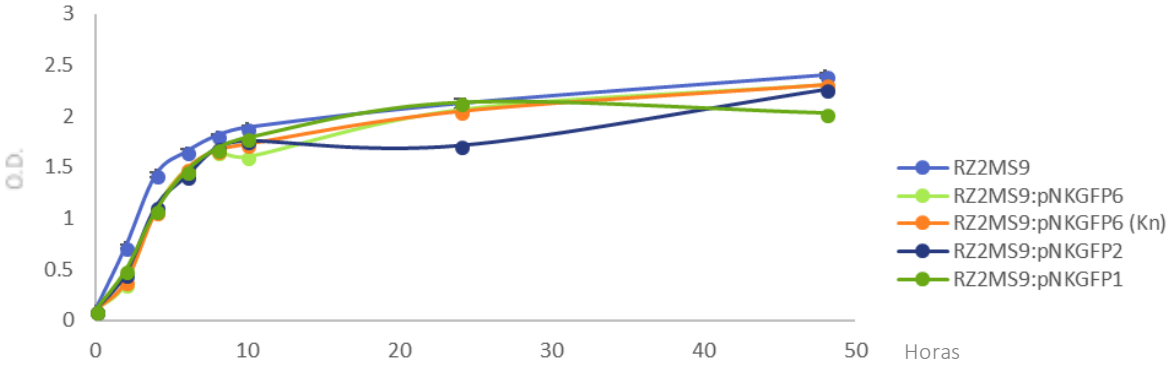

Figure 2. Growth curve was constructed using tree different RZ2MS9 GFP-tagged mutants: RZ2MS9::pNKGFP1, RZ2MS9::pNKGFP2, RZ2MS9::pNKGFP6 and the RZ2MS9 WT. All strains were growth in 50 ml of LB broth medium, only the RZ2MS9::pNKGFP6 was also tested in LB supplemented with 50 µg.ml^{-1} of kanamycin (Kn). All strains were evaluated in triplicate. The Optical Density (OD_{600nm}) was measured in spectrophotometer (Ultraspec 3000 Amersham Pharmacia Biotech) every 2 hours, for 12 hours, and then 24 and 48 hours after inoculation.

2.3.2. Maize co-colonization by GFP-tagged RZ2MS9 and RFP-tagged Ab-v5

None fluorescent bacterial cells were observed in non-inoculated plants at 15th and 30th DAG. Maize samples showed cellular auto-fluorescence, however, the presence of few RZ2MS9::pNKGFP6 and Ab-v5::pWM1013 cells fluorescing in bacterial aggregations were clearly visible. RZ2MS9::pNKGFP6 was able to colonize endophytically roots and leaves of maize, demonstrating its endophytic behavior (Figure 4).

In leaves, RZ2MS9::pNKGFP6 cells were mostly observed in chlorenchyma, in both palisade and spongy parenchyma. It was also possible to observe bacterial cells colonizing the sub-stomata chamber, epidermal cells and in xylem vessels (Figure 4). RZ2MS9::pNKGFP6 was most frequently observed when inoculated alone, than co-inoculated with Ab-v5::pWM1013. Few RZ2MS9::pNKGFP6 cells were found in maize root cells (Figure 4). However, Ab-v5::pWM1013 was not observed colonizing maize roots.

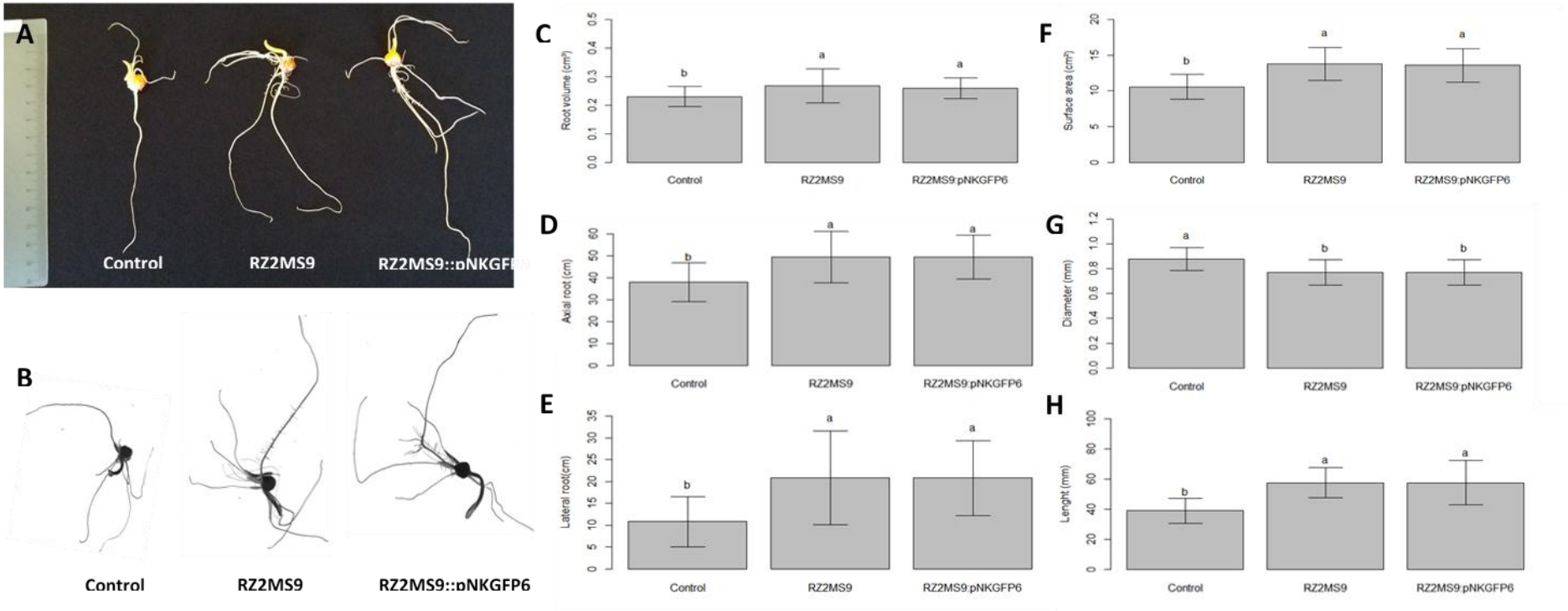

Figure 3: *In vitro* seed germination test, using the hybrid P4285H DuPont® Pioneer® maize with tree treatments: Non-inoculated control, RZ2MS9 WT and RZ2MS9::pNKGFP6. The evaluation was performed 7 days after bacterization, in towel paper. **(A)** Picture of germinated maize seeds, 7 days after bacterization; **(B)** Images of germinated maize seeds captured at 400 dpi resolution with the Epson® Expression 11000XL scanner. The images were analyzed by the software WinRHIZO Arabidopsis, and the parameters evaluated and statistically analyzed were: **(C)** Root volume; **(D)** Axial root length; **(E)** Lateral root length; **(F)** Surface area; **(G)** Average diameter; **(H)** Total length. Different letters represent significant different means according to Tukey's test.

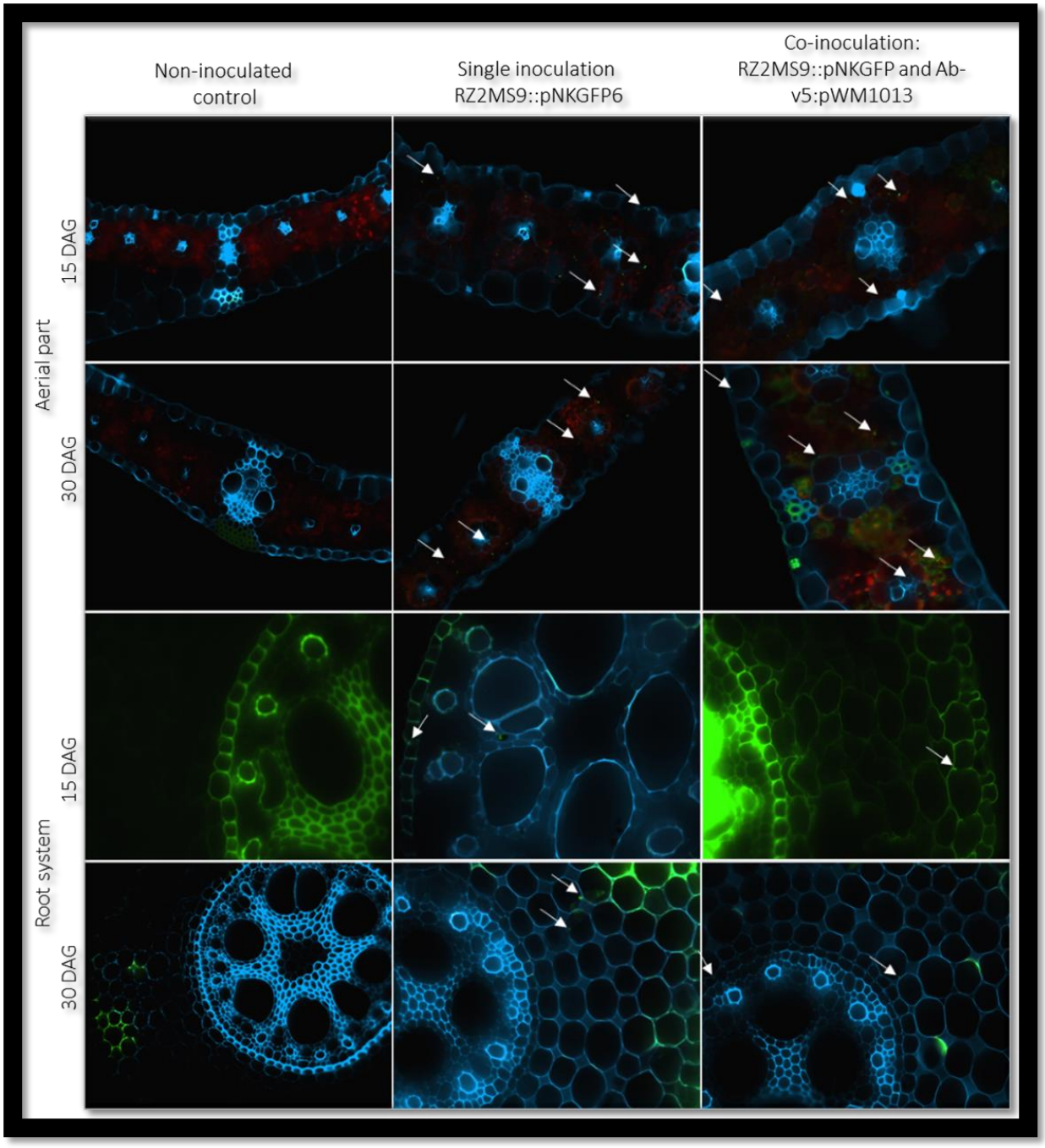

Figure 4. RZ2MS9::pNKGFP6 and Ab-v5::pWM1013 maize colonization through fluorescence optical microscopy. The cross sections were performed in the aerial part and root system of maize, 15th and 30th DAG. No fluorescent bacterial cells were observed in the non-inoculated control. The arrows indicate the presence of the bacterium RZ2MS9::pNKGFP6. The magnification used was 200X.

2.3.3. Monitoring of RZ2MS9::pNKGFP6 and Ab-v5::pWM1013 maize cross-colonization by qPCR

We observed a higher number of RZ2MS9::pNKGFP6 cells inner root and leaves maize at 15 DAG comparing with 30 DAG. The co-inoculation with Ab-v5::pWM1013 did not affect the R2MS9::pNKGFP6

maize colonization, except at 15 DAG in maize leaves. The RZ2MS9::pNKGFP6 moved from maize roots to aerial parts, increasing the bacterial cell density in aerial tissues comparing to root tissues independently of the Ab-v5::pWM1013 co-inoculation. In general, it was observed a decreasing of RZ2MS9::pNKGFP6 cell density comparing 15 and 30 DAG (Figure 5).

The qPCR performed using the primers AzoR2.1F and AzoR2.1R to monitor Ab-v5::pWM1013 quantification was not efficient. Using the DNA of root system and the shoot, in both evaluated times, 15 and 30 DAG, Ab-v5::pWM1013 was not detected.

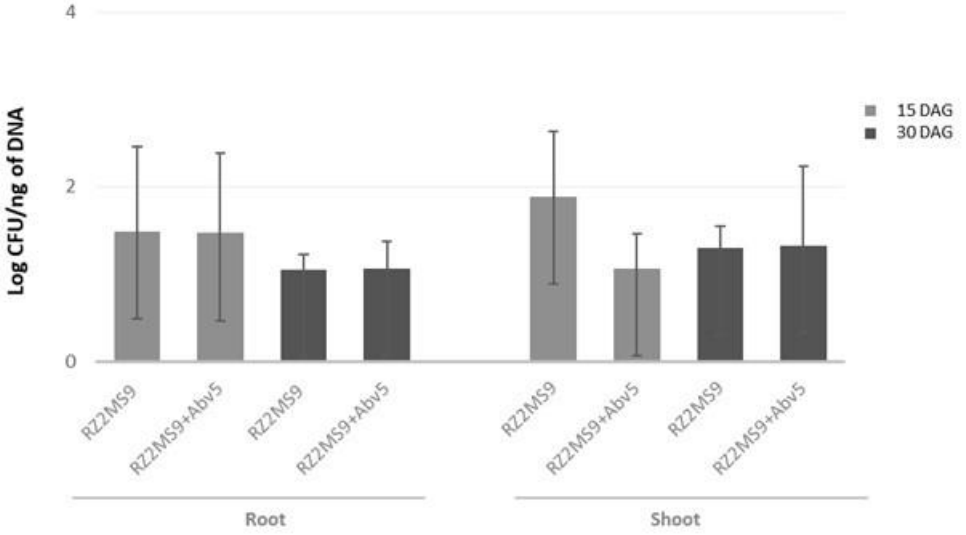

Figure 5. *Bacillus* sp. RZ2MS9::pNKGFP6 density during maize cross-colonization, measured using qPCR at 15 and 30 DAG. The quantification was performed in plants under single inoculation of RZ2MS9::pNKGFP6 and co-inoculated with RZ2MS9::pNKGFP6 and Ab-v5::pWM1013. The abundance data, in CFU/ng of DNA, were log transformed to stabilize the variance. The results are the means of the three replicates for each sample. The bars represent the standard error of each treatment.

2.3.4. Maize growth promotion assay

Eleven parameters related with maize growth were evaluated. However, six of them: stem diameter, root length, fresh weight of shoot and root, surface area, lateral and axial root length, did not differ according to the treatments. Root and shoot dry weight were improved by the co-inoculation of RZ2MS9 and Ab-v5 (Figure 6A and 6B). Compared to control, the co-inoculation increased in 50.8 % and 79.6 % the root and shoot dry weight respectively. Plants height was similar to RZ2MS9 single inoculation and co-inoculated with Ab-v5 (Figure 6C). Root volume and root diameter were also increased in 33.9 and 12.5 % by the co-inoculation compared with the control (Figure 6D and 6E). It was clear the improvement in maize root architecture due to bacterial inoculation (Figure 7).

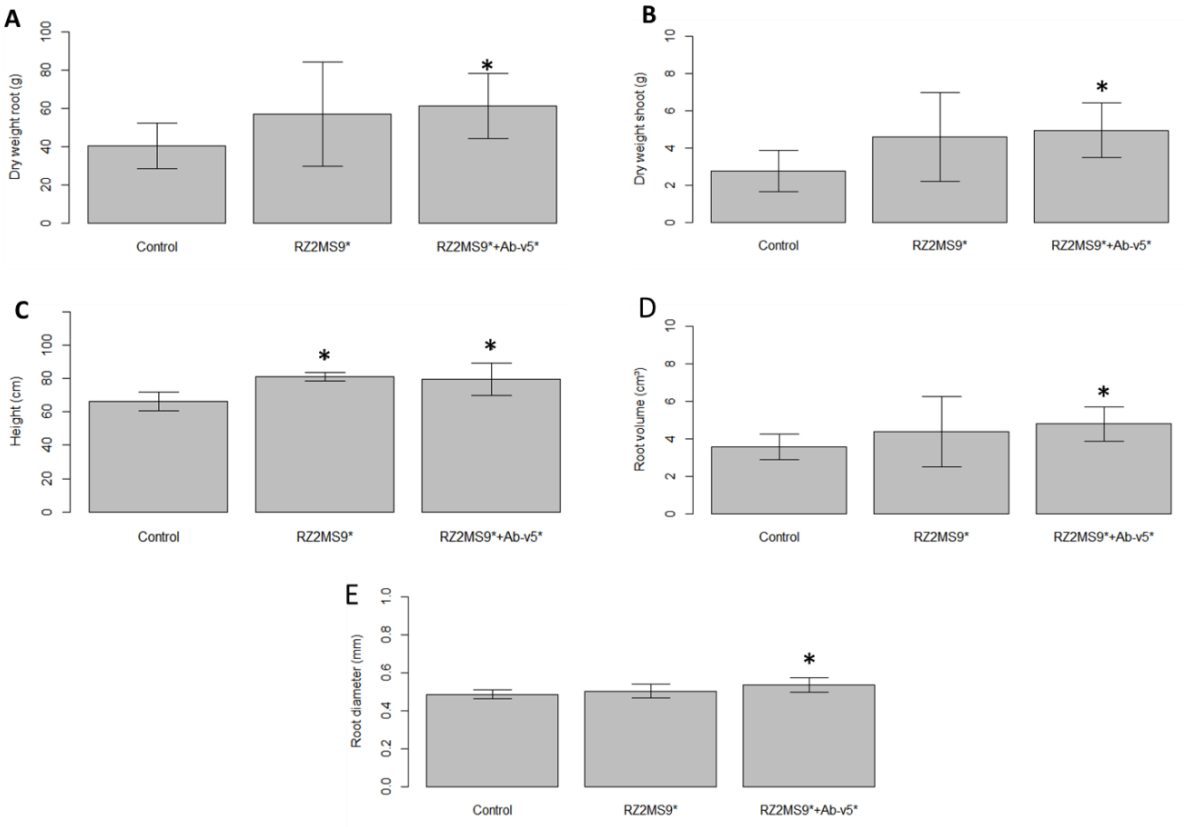

Figure 6. Evaluated parameters of maize plants response by the inoculation of *Bacillus* sp. RZ2MS9::pNKGFP6 (RZ2MS9* in the graphic) and under co-inoculation of RZ2MS9::pNKGFP6 and *A. brasilense* Ab-v5::pWM1013 (RZ2MS9*+Ab-v5*), at 30 DAG. Data represent the means of 6 plants per treatment and the symbol (*) represents the statistically different values, when compared to the non-inoculated control, by the T test at 1% of significance level. The evaluated parameters were: **(A)** Dry weight of the root system; **(B)** Dry weight of shoot; **(C)** Shoot height; **(D)** Root volume; **(E)** Root diameter.

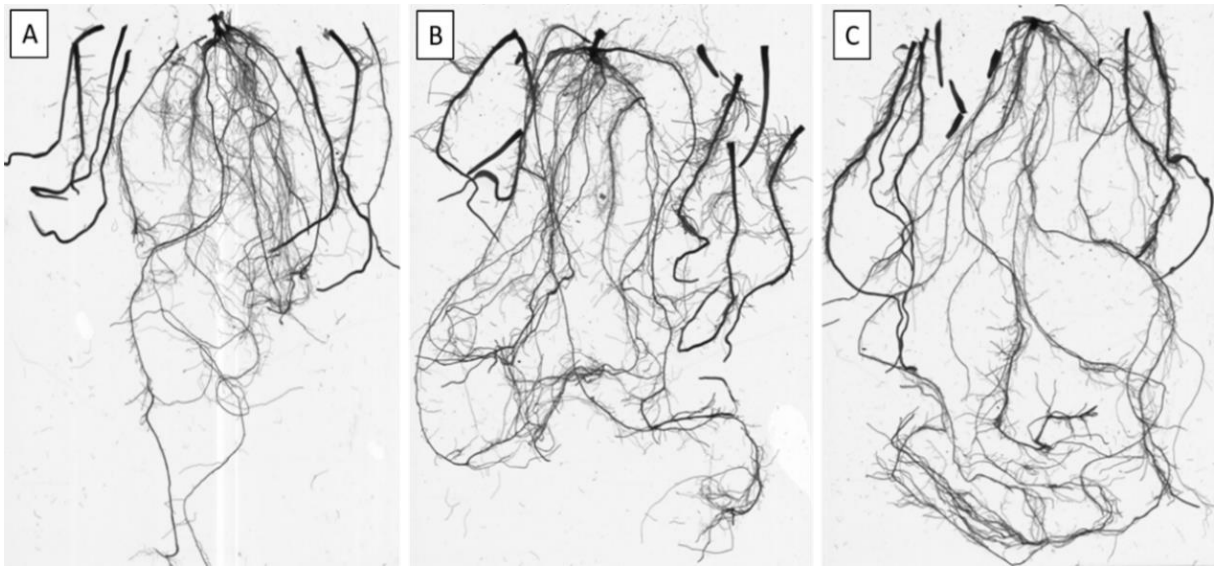

Figure 7: Root images of maize architecture obtained by Epson® Expression 11000XL scanner used to measure the evaluated parameters of maize root by the inoculation of *Bacillus* sp RZ2MS9::pNKGFP6 and under co-inoculation with *A. brasilense* Ab-v5::pWM1013, 30 DAG. **(A)** Maize root, non-inoculated control; **(B)** Maize root inoculated with RZ2MS9::pNKGFP6; **(C)** Maize root co-inoculated with RZ2MS9::pNKGFP6 and Ab-v5::pWM1013.

2.4. DISCUSSION

Electrotransformation has been chosen to target PGPB aiming their monitoring during plant interaction (Compant et al., 2005; Gai et al., 2009; Quecine et al., 2012; Dourado et al., 2013). The protocol proposed by Schurter et al. (1989) was efficient to target *Bacillus* sp. RZ2MS9, using integrative and replicative plasmids. Interestingly, the number of RZ2MS9 transformants obtained with plasmid pNKGFP was larger than obtained with plasmids pBAV1k-T5gfp. The lower transformation efficiency with the plasmid pBAV1k-T5gfp may be due to the type of this plasmid replication, called the rolling-circle. However, it has been found in Gram-positive bacteria and has been previously reported to target *Bacillus* spp. (Bryksin and Matsumura, 2010).

In general, transformants amount obtained in this work was lower than obtained to *Bacillus thuringiensis*, 10^7 transformants per µg of DNA, using similar protocol (Schurter et al., 1989). The authors used pBC16, a natural plasmid isolated from *B. cereus*, described as stable in others *Bacillus* strains (Bernhard et al., 1978). Furthermore, some protocols have been published to target *Bacillus* spp. (Bone and Ellar, 1989; Lereclus et al., 1989; Mahillon et al., 1989; Masson et al., 1989; Schurter et al., 1989), showing a range between 10^2 to 10^7 transformants per µg DNA. *Bacillus* sp. RZ2MS9 amount of transformants obtained was in average 10^3 transformants per µg of plasmid DNA, a value expected for this genus.

The pNKGFP integrative plasmid was constructed by Ferreira et al. (2008) to target *Pantoea agglomerans* strain 33.1, to study the colonization of *Eucalyptus* seedlings. This plasmid was described as an efficient GFP-target for bacteria able to promote the plant growth, by Quecine et al. (2012) as well to target the phytopathogen *Leifsonia xyli* subsp. xyli, a Gram-positive bacterium (Quecine et al., 2016). Similarly, this plasmid was efficient to target *Bacillus* sp. RZ2MS9, a Gram-positive plant growth-promoting bacterium.

The transformant RZ2MS9::pNKGFP6 was selected because it was unique one integrative copy (data did not show) and demonstrated an indistinguishable from the wild-type strain concerning bacterial growth rate. At the first time, it was evaluated the effects of RZ2MS9 and RZ2MS9 GFP-tagged on maize seed germination. The bacterial presence did not show a direct influence on the number of germinated seeds. Although, RZ2MS9 and RZ2MS9::pNKGFP6 inoculation improved maize root architecture when compared to the non-inoculated control.

Different mechanisms should be involved in plant growth promotion. The improvement on maize root architecture during seed germination test, as well the plant growth promotion assays, can be related with the carbon/nitrogen balance. It is known that the carbon/nitrogen balance seems to be crucial for the regulation of gene expression of pathways related to seed germination and plant development (Osuna et al., 2015). In RZ2MS9 genome draft was identified 14 genes related to nitrogen

metabolism, among them there is possible highlighted the nitric oxide synthase oxygenase (*nos*) that catalyze the production of nitric oxide (NO), as well the *nirC* gene (nitrite transporter), which catalyzes nitrite uptake and nitrite export across the cytoplasmic membrane (Batista et al., 2016). In *Arabidopsis*, the NO was described as germination inducer and as able to reduce primary root growth and induce lateral root development (Osuna et al., 2015). Moreover, *Bacillus* sp. RZ2MS9 as previously described as IAA producer (Batista et al., 2018), an essential phytohormone for the root development (Osuna et al., 2015). We also observed the induction of maize lateral root development by *Bacillus* sp. RZ2MS9 inoculation. Besides that, the IAA behave as a regulatory signal for other important mechanisms related to plant metabolism.

Ryu et al. (2003) demonstrated that airborne volatiles from growth-promoting strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* can stimulate the growth of *A. thaliana* seedlings, these volatiles were described as compounds that stimulate root system development (Gutiérrez-Luna et al. 2010). The genes *alsS* and *alsD,* related with those volatiles production pathway, were detected in the genome of *Bacillus* sp. RZ2MS9 (Batista, 2017). *alsS* and *alsD* gene expression may also be related to the greater development of the roots in the germination of maize seeds. Thus, the production of NO, IAA and volatile compounds by this strain should be better investigated.

The fluorescence microscopy and the qPCR revealed that the RZ2MS9::pNKGFP6 strain was able to colonize maize plants, demonstrating its endophytic behavior. *Bacillus* sp. RZ2MS9 has been isolated from rhizosphere of guarana (Batista et al., 2018). However, such as RZ2MS9, several bacteria described as rhizobacteria are able to colonize plant tissues (Compant et al., 2005; Hardoim et al., 2008). RZ2MS9::pNKGFP6 was able to penetrate plant roots and migrated through xylem to shoot. Similar to the results obtained by Compant et al. (2005), that observed the colonization of *V. vinifera* L. plantlets by *Burkholderia phytofirmans* PsJN, that demonstrated the ability to colonize the rhizoplane, roots, stems, and leaves. Hao and Chen (2017) GFP-targeted the PGPR *Paenibacillus polymyxa* strain WLY78 and evaluated its colonization in maize. The authors observed that this strain was able to colonize the whole plant and tracked into the cells of the roots, vascular system, and leaves.

Different strains of *A. brasilense* were described as an internal root colonizer or only able to colonize the root surface (Pereg et al., 2016). Tschoeke (2016) studying *A. brasilense* Ab-v5::pWM1013 during maize colonization observed an endophytic behavior of this bacterium. He observed Ab-v5::pWM1013 colonizing the root cortical parenchyma and the leaf mesophyll. Under co-inoculation with *Burkholderia ambifaria* RZ2MS16, Ab-v5::pWM1013 showed the same ability to colonize maize plants, been observed in the leaves and inside of roots (Tschoeke, 2016). In the present work, Ab-v5::pWM1013 was not observed colonizing maize plants in presence of RZ2MS9::PNKGFP. The absence of Ab-v5::pWM1013 observation suggested the interaction between these strains, resulting in a modification of Ab-v5::pWM1013-maize interaction. Although, *in vitro* assays were performed and the

co-cultivation between *Bacillus* sp. RZ2MS9 and *A. brasilense* Ab-v5, did not show any type of antagonism (data not shown). The possible differences during bacteria interaction *in vitro* and *in vivo* can be result of the complexity of the analyzed system and due to the numerous environmental factors that effect in the results of inoculation and consequently bacteria-plant interaction (Pereg et al., 2016).

By qPCR and FM we clearly demonstrated that *Bacillus* sp. RZ2MS9::pNKGFP6 was able to co-colonize the same niches previously described as colonized by *A. brasilense* Ab-v5::pWM1013 in maize (Tschoeke, 2016). The possible competition between RZ2MS9 and Ab-v5 for the plant colonization in the start of seed germination may be the answer of why Ab-v5::pWM1013 was not observed in the co-inoculated plants. During a niche competition, bacteria present different strategies, one of which is the ability to interfere in the communication process of the other bacteria, system called quorum quenching (QQ) (Bassler, 2002). The bacterial communication, known as quorum sensing (QS), is used for many microorganisms to regulate the gene expression of them and enhance survival of the microorganism (Safari et al., 2014). *Bacillus* sp. RZ2MS9 is able to produce the enzyme AHL-lactonase, that degrade the QS molecules of Gram-negative bacteria, as the produced by *A. brasilense* Ab-v5 (Almeida et al., 2018 unpublished). The production of this enzyme can be directed related with the alteration of Ab-v5::pWM1013 and RZ2MS9::PNKGFP during maize colonization.

The non-detection of *A. brasilense* Ab-v5::pWM1013 through the qPCR technique corroborates the hypothesis of the initial interaction between Ab-v5::pWM1013 and RZ2MS9::PNKGFP6. Tschoeke (2016) observed that Ab-v5::pWM1013 showed preference for colonization of maize rhizosphere, whereas the evaluation performed 30 DAG, the number of bacteria Ab-v5::pWM1013 obtained by re-isolation showed decrease in the aerial part and increase in rhizosphere region. Therefore, in addition to the probable initial interaction between Ab-v5::pWM1013 and RZ2MS9::PNKGFP6, the preference of Ab-v5::pWM1013 to colonizing the rhizosphere region over time (not evaluated in this study) corroborates the non-detection of Ab-v5::pWM1013 in maize plants. Although, the better results obtained in plant promotion assay in co-inoculated plants, demonstrates the occurrence of synergistic interaction between these bacteria and maize at least in first moments during the interaction.

At first time, we clearly demonstrated that RZ2MS9 and Ab-v5 maize co-inoculation presented better results than single inoculation with RZ2MS9. Our data corroborate previous studies that demonstrated the synergistic effects of co-inoculation in the plant development (Chauhan and Bagyaraj, 2015; Korir et al., 2017; Molina-Romero et al., 2017; Santiago et al., 2017). Chauhan and Bagyaraj (2015) demonstrated that co-inoculation of *P. polymyxa, P. agglomerans* and *Funneliformis mosseae* gave better results in growth and yield parameters of French bean. Korin et al. (2017) demonstrated that co-inoculation of PGPR (*P. polymyxa* and *Bacillus megaterium*) and Rhizobia (IITA-PAU 987, IITA-PAU 983 and CIAT 899) has a synergistic effect on common bean (*Phaseolus vulgaris* L.) growth. The plant growth of potato seedlings using co-inoculated strains was demonstrated by Santiago et al. (2017). The authors

used *Streptomyces* sp. R170 under co-inoculation with *Sphingomonas* sp. T168 or with *Methylibium* sp. R182, revealed by the improved production of IAA and siderophores, which may have caused the significant increase observed in the weights of potato seedlings. The inoculation of maize with an arbuscular mycorrhizal fungus in consortium with PGPB showed increasing in the biomass production and in the leaves mineral nutrient content (Zoppellari et al., 2014). Also, in maize the inoculation of four PGPB (*Pseudomonas putida* KT2440, *Sphingomonas* sp. OF178, *A. brasilense* Sp7 and *Acinetobacter* sp. EMM02) demonstrated higher values of plant growth parameters compared to mono and the non-inoculated plants (Molina-Romero et al., 2017).

The parameters: dry weight, shoot height, volume and the diameter of roots clearly demonstrated significant improvement comparing the co-inoculation and non-inoculated control. Supporting our result, Cassán et al. (2008), observed that the co-inoculation of seed maize with *A. brasilense* Az39 and *Bradyrhizobium japonicum* E109 resulted in the improvement of shoot length, shoot and root dry weight. However, Felici et al. (2008) observed worse results co-inoculating *B. subtilis* 101 and *A. brasilense* Sp245 in tomato comparing with single inoculation. Thus, it is clear the importance of preliminaries studies concerning the co-inoculation as a feasible alternative of biofertilizer application.

Additionally, colonization studies have fundamental importance to understand the interaction between PGPB and the interested crop. Bacteria tagged with different fluorescent proteins can support to understand the action of each strain during host colonization. This strategy allows to verify the best form of application or re-application of each bacteria. In view of these, this work demonstrated the synergistic interaction between *Bacillus sp.* RZ2MS9::PNKGFP and *A. brasilense* Ab-v5::pWM1013 during maize colonization, being the co-inoculation of these strains the best strategy to maize growth promotion. The increase of root and shoot dry weight, root volume and diameter in the co-inoculated plants it was resulted of a synergistic combination of the mechanisms of these strains to promote plant growth, showing that inoculation with more than one strain can be a good choice to development of bio-fertilizers. Although, future efforts will be necessary to determine which mechanisms are involved in such interaction, and once these strains are not specific crop, the synergistic interaction between *Bacillus sp.* RZ2MS9 and *A. brasilense* Ab-v5 can be evaluated in other cultures.

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3. THE QUORUM QUENCHING SYSTEM IN A PLANT GROWTH-PROMOTING *BACILLUS* SP. RZ2MS9

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ABSTRACT

Quorum Sensing (QS) is an important cell-cell communication system that allows bacterial cells to recognize their own population density by sending and detecting extracellular signals. These signals are recognized by outer membrane receptors and alter the expression of specific genes, regulating a wide variety of important biological functions. The molecules involved in this communication may be also detected by other bacterial species and plants, inducing a modulation of their gene expression, metabolism and growth. In response to interspecific detection ability of these molecules, the quorum quenching (QQ) system has been evolved in some bacteria as competitive advantage for niches colonization. The QQ is involved in the general control of the physiology of bacterial populations, interfering in the cell-cell communication by degrading the QS molecules, demonstrated how an adaptive advantage in niches in which different bacterial populations compete for resources. The *aiiA* gene, was one of the first gene related with the QQ system in *Bacillus* sp. This gene encodes the enzyme AiiA lactonase, that is able to hydrolyze the lactone bond of QS molecules from Gram-negative bacteria, called *N*-acyl homoserine lactone (AHL). In the annotation of genome draft of *Bacillus* sp. RZ2MS9, the *aiiA* gene presence was detected and the AHL degradation ability was confirmed using a new QQ biosensor strain, *Agrobacterium tumefaciens* At11006 and validated by *A. tumefaciens* NTL4. The confirmation of AiiA lactonase function was confirmed by knockout of the *aiiA* gene, using the newest genome editing technique CRISPR-Cas9 system.

Keywords: *PGPB; Biosensor; CRISPR-Cas9; knockout; aiiA; AHL-lactonase*.

3.1. INRODUCTION

Initially, it was believed that quorum sensing (QS) was just a cell-cell communication system that allowed bacterial cells to recognize their own population density by sending extracellular signals, named

auto-inducers, that were recognized by outer membrane receptors. With the increasing of bacterial population, the concentration of these molecules also increases in the extracellular environment and, consequently, the cells perceive the bacterial density altering the expression of specific genes, regulating a wide variety of biological functions, such as plasmid transfer, virulence induction genes, antibiotic production, motility and biofilm formation (Bassler, 2002; Dong et al., 2000). In addition, the molecules involved in this communication may be also detected by other bacterial species and plants; inducing a modulation of their gene expression, metabolism and growth (Ortíz-Castro et al., 2009).

The best-known QS system is from Gram-negative bacteria, regulated by the production of auto-inducers from acyl homoserine lactones family (AHLs) (Ammor et al., 2008). This system was first described in the bioluminescent marine bacterium *Vibrio fischeri*, being responsible for regulation of light production (Ruby, 1996). Since then, other types of signaling molecules have been identified (Barber et al., 1997; Flavier et al., 1997) for instance, the furanosyl borate diester that appears to be involved in interspecific bacterial communication (Chen et al., 2002).

Quorum quenching (QQ) is a system also involved in the general control of the physiology of bacterial populations. However, QQ molecules interfere with cell-cell communication by degrading the QS molecules. This system of degradation of QS molecules is important in niches where different bacterial populations compete for resources, since the capacity for detection of QS molecules of another bacterial species is an adaptive advantage (Waters and Bassler, 2005). There are three known classes of enzymes able to degrade AHL signals: the lactonases, the acylases and the oxidoreductases. The most class studied is the AHL lactonase, described for the first time in 2000 (Dong et al., 2000), when described the gene *aiiA* (auto-inducer inactivation gene) from *Bacillus* sp., able to inactivate several different types of AHL.

Many *Bacillus* species have QQ systems described (Dong et al., 2002; Lee et al., 2002). *Bacillus* spp. can secret an enzyme known as AiiA lactonase (AHL lactonase), encoded by the *aiiA* gene. This enzyme can hydrolyze the ester bond of the homoserine lactone ring (Dong et al., 2002), thus the QS molecules of Gram-negative bacteria are altered, and consequently QS-dependent processes are not activated. The QS regulation of virulence seems to be one of the common strategies that many bacterial pathogens have adopted during evolution to ensure their survival in host-pathogen interactions. This kind of QQ strategy could have wide applications as an alternative method to control bacterial phytopathogens (Dong et al., 2001). So, the QQ has been proposed as a novel biocontrol strategy against plant pathogens, as well against the human pathogens, once the AHLs regulate genes critical for dissemination and virulence, given the pivotal role QS plays in pathogenic interactions (Alt-Morbee et al., 1996; Loh et al., 2002). The inactivation or degradation of AHLs via QQ presents an attractive target for designing innovative disease control strategies (Molina et al., 2003).

The lactonases are metalloproteins that show the ability of hydrolyzing the ester bond of the AHL ring of 3OC8HSL to produce *N*-(3-oxooctanoyl)-L-homoserine (Zhang et al., 2002), this way they generally have very broad AHL substrate specificity, due to the fact that homoserine lactone ring is conserved among all AHLs (Liu et al., 2008). A diverse groups of bacteria present species able to inactivate AHLs enzymatically as *Agrobacterium, Sphingomonas, Ochrobactru*m and *Bosea* of the α-Proteobacteria group, in the β- Proteobacteria the genus *Variovorax, Ralstonia* and *Comamonas* can be observed, among the γ-Proteobacteria *Pseudomonas*, *Acinetobacter* and in the Firmicutes, the genus as *Bacillus* and *Arthrobacter* was related (Uroz et al., 2009).

Studies about the role of QS and QQ in promoting plant growth are incipient. The need for a minimum level of initial inoculum to plant growth-promoting bacteria (PGPB) to promote plant growth, strongly supports the idea that bacterial QS plays an important role in plant-PGPB interactions (Persello-Cartieaux et al., 2003). Not only bacteria can use the QQ system, but also plants can produce QS signal mimics or QS-interfering molecules and result in the QQ (Teplitski et al., 2000; Zhang et al., 2017). Some plants, such as soybean, rice and tomato, are able to secrete compounds having AHL-mimicking activities (Teplitski et al., 2000; Rasmussen et al., 2005; McDougald et al., 2007), these mimicking compounds seem to be important in determining the outcome of interactions among plants and a diverse range of pathogenic microbes (Zhang et al., 2017).

The present work aimed to study the QQ system in *Bacillus* sp. RZ2MS9, a known plant growth promotion bacteria (PGPB) that present the *aiiA* gene annotated in your genome (Batista et al., 2016, Batista et al., 2018). The AHL lactonase activity was confirmed using a new QQ biosensor strain, *Agrobacterium tumefaciens* At11006, constructed in this work, and validated by *A. tumefaciens* NTL4. The *aiiA* gene was efficiently knockout using the technique CRISPR-Cas9 system (Jiang et al., 2015), and you action in the QQ system confirmed.

3.2. MATERIAL AND METHODS

3.2.1. Bacterial strains and Culture conditions

The bacterial strains used in this work were shown in Table 1. Cell overnight culture was mixed in equal parts with DMSO diluted to 14 % in LB medium, placed in polypropylene 2 ml vials and stored at - 80°C.

Table 1: Bacterial plasmids and strains used in this work

Plasmids	Description	Reference
pJH110	*E. coli* DH5α with the plasmid pJH107 containing rfp-attB fragment - Kn^R and Gm^R	Heil et al., 2012
pJH111	*E. coli* DH5α with pJH110 plasmid with *traI::lacZ* gene fusion - Kn^R and Gm^R	This work
pCas	Cas9 enzyme; pCas promoter; RepA101 Kn^R	Jiang et al., 2015
pTargetF	pJ23119 promoter; pMB1 replicon - Sp^R	Jiang et al., 2015
Strains	**Description**	**Reference**
A. tumefaciens At11006	C58 with the fusion *traI::lacZ* - Rf^R and Gm^R	This work
A. tumefaciens C58	Wild-type strain	Goodner et al.,2001; Wood etal., 1997
A. tumefaciens NTL4	*pZLR4 – TraR* promoter and the fusion *traG::lacZ* Gm^R and carbenicillin ^{R}	Szenthe and Page, 2003
E. coli pRK600	*E. coli* DH5α with the Mobilization plasmid pRK600 - Cm^R	Finan et al., 1986
E. coli DH5α	F^- supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 (ϕ80lacZ ΔM15)	Hanahan,1983
E. coli W3110	Wild-type strain - Rf^R	ATCC® 27325™
E. coli pJC2	*E. coli* DH5α with pRK7813 containing ϕC31 integrase under control of lac promoter - Tc^R	Constructed by Dr. J. Cheng 68381. Heil, 2015
Bacillus sp. RZ2MS9	Plant growth promoter bacteria isolated from guarana	Batista et al., 2018
Pantoea agglomerans 33.1	Plant growth promoter bacteria isolated from *Eucalyptus grandis*	Plaza et al., 2004
Azospirilum brasilense Ab-v5	Plant growth promoter	Hungria et al., 2010

Escherichia coli, Agrobacterium tumefaciens strains and *Bacillus* sp. RZ2MS9 were grown in LB medium (Sambrook and Russell, 1989) at 37°C, 30°C and 28°C respectively. The *Azospirillum brasilense* Ab-v5 was grown in DYGS medium at 28°C (Rodriguez et al., 2004). For *E. coli*, antibiotics were used at the following concentrations: 25 $\mu g.ml^{-1}$ of kanamycin sulfate (Kn); 25 $\mu g.ml^{-1}$ of gentamicin sulfate (Gm); 10 $\mu g.ml^{-1}$ of tetracycline hydrochloride (Tc); 10 $\mu g.ml^{-1}$ of chloramphenicol (Cm); 50 $\mu g.ml^{-1}$ of rifampicin (Rf). For *A. tumefaciens* strains the antibiotics concentration used was: 25 $\mu g.ml^{-1}$ of Kn; 25 $\mu g.ml^{-1}$ of Gm; 200 $\mu g.ml^{-1}$ of streptomycin sulfate (Sm); 100 $\mu g.ml^{-1}$ of spectinomycin dihydrochloride (Sp); 10 $\mu g.ml^{-1}$ of Cm; 50 $\mu g.ml^{-1}$ of Rf. For the QS assays was used of 50 $\mu g.ml^{-1}$ of x-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) on the culture medium.

3.2.2. Construction of *A. tumefaciens* At11006 QQ biosensor

For construction of a QQ biosensor strain, using the QS system of *A. tumefaciens*, the first step was the construction of the plasmid pJH111 with the translational fusion of the genes: *traI* and *lacZ.* After that, this gene fusion was inserted in *A. tumefaciens* C58 by tretraparental mating.

3.2.2.1. Genomic DNA extraction

The strains *A. tumefaciens* C58 and *E. coli* W3110 were grown in 5 ml of LB medium at 30° and 37°C respectively, for 12 hours. The cultures were added to a 1.5 ml microtube and the cells were pelleted by centrifugation at 19000x g for 2 minutes. The supernatant was decanted, and the pellet resuspended in 200 µL of 0.2M NaCl and 20 µL of 0.2M EDTA, 10 µL of 10 % SDS solution and 5 µL of 10 µg/ml pronase were added and then incubated at 70°C for one hour. After that, 300 µL of chloroform was added and the tube vortexed. The tubes were centrifuged at 19000x g for 10 minutes and the aqueous layer dispense into a new microtube. 450 µL of ice cold 95 % ethanol was added, the samples were vortexed and after centrifuged at 19000x *g* for 15 minutes, and the supernatant removed. Were added 1 ml of 70 % ethanol to wash pellet and then centrifuged 19000x g for 15 minutes. The liquid was removed, and the pellet dried at room temperature between 10-20 minutes, and the pellet was resuspended in 50 µL of 2mM tris.

3.2.2.2. Construction of the translational fusion

The gene *lacZ* and *traI* was obtained by PCR amplification using the primers described in Table 2, using the genomics DNAs of *E. coli* W3110 and *A. tumefaciens* C58 as template, respectively. For the fusion of the amplicons, the overlap extension PCR was used. For this technique, the primers have specific characteristics reverse primer of the first gene must have in your 5' region 20 pb inverted complement of the 5' region of the forward primer of the second gene. For the cloning into a vector, the forward primer of the first gene and the reverse primer of the second gene present the restriction site for the *Pae*I enzyme.

For the *traI* gene amplification, the reaction was performed using the Xtreme KOD hot startDNA polymerase (Novagen®) using: 5 µl of dNTP (2 µM); 12.5 µl of 2X Xtreme buffer; 0.75 µl of primer TraI_F (10 µM); 0.75 µl of primer TraI_R (10 µM), 0.5 µl of Xtreme KOD DNA polymerase, 4.5 µl of water and 1.0 µl of DNA, 25 µl per reaction. The thermal cycling protocol used was: 2 minutes heating step at 94°C; 30 cycles were as follows: 98°C for 10 seconds for denaturing, 59°C for 30 seconds for annealing, and

68°C for 3 minutes for extension. And a final extension set, 68°C for 5 minutes. For the gene *lacZ* amplification, the same conditions describe above were used, using the primers LacZ_F (10 µM) and LacZ_R (10 µM) and as template the genomic DNA of *E. coli* W3110.

The fusion of this genes was performed by PCR reaction, using as template the PCR products *TraI* and *LacZ*. For this, the primers TraI_F and LacZ_R were used, in the same conditions described above, but using 50°C as annealing temperature.

3.2.2.3. Vector extraction

A single colony of the *E. coli* (pJH110) was inoculated into 5 ml of LB medium in a 16 mm test tube with Kn and incubated for 48 hours at 30°C with constant shaking (200 rpm). The bacterial cells culture was centrifuged at 19000x g for 2 minutes. The cell pellet was resuspended in 250 ml of buffer P1 (100 mM Tris-HCl ph 8.0, 10 mM EDTA, 100 µg/ml RNAse A). To lyse the cells 250 ml of buffer P2 (0.2 M NaOH, 1 % SDS) was added. Then, 350 ml of buffer N3 (4.2 M Guanidine thiocyanate, 0.9 M potassium acetate, pH to 4.8 with acetic acid) was added. The tube was immediately inverted several times to ensure quick and even neutralization. The tube was then centrifuged for 6 minutes at 19000x g to a silica spin column (Bio Basic, Markham Canada). The column was centrifuged at 19000x g for 1 minute. The flow through was discarded and 500 ml of wash buffer (75 % ethanol and 10 mM tris-HCl pH 7.5) was added. Centrifugation was repeated and the flow through was discarded. After, the centrifugation was repeated to dry the column. 35-50 µl of 2mM tris-HCl elution buffer was added to the column and allowed to incubate at room temperature for 2 minutes. The column was placed in a 1.5 ml centrifuge tube and centrifuged as above. The flow through of this step was the final product, and it was either used immediately or stored at - 20°C.

3.2.2.4. Vector assembly and transformation

The plasmid pJH110 and the PCR product *traI::lacZ* were digested using the enzyme *Pae*I, Fast Digest (Thermo Fisher Scientific Inc.) according to the protocol suggested by the manufacturer. After the digestion, they were purified by gel extraction. The ligation reaction between the vector pJH110 and the PCR product was performed by the enzyme T4 DNA Ligase (Thermo Fisher Scientific Inc.), giving rise to pJH111 plasmid.

The vector insertion in *E. coli* DH5α was performed using 10 µL of the ligation added to the chilled tubes, and 100 µl of thawed ice-cold DH5α competent cells were added. The DNA cell mixture was allowed incubate on ice for 30 minutes and after placed into a 42°C in water bath for 90 seconds,

and then the tubes were placed back on ice for 5 minutes. So, 1 ml of LB broth was added and the tubes incubate at 37°C for 1 hour. The cells were pelleted by spinning at max speed in a microcentrifuge for 30 seconds. The supernatant was poured off, leaving approximately 50-100 µl in the tube. The cells were resuspended in the reduced volume and the cells were spread plated on LB medium containing Kn and x-gal and incubated at 37°C for 24 hours.

The transformants that presented Kn resistance and blue color were selected and passed for a 5 ml of LB liquid with Kn and grown in shaker for 12 hours and the plasmid extraction was performed by the protocol described above.

The presence of gene fusion *traI::lac*Z in the plasmid pJH111 was verified by restriction digestion with the enzyme *Pae*I and by PCR, using the primers TraI_F and LacZ_R under the same conditions described above.

3.2.2.5. Construction of the *A. tumefaciens* biosensor

The plasmid pJH111 (pJH110 with *traI::lacZ)* was inserted *A. tumefaciens* strain C58 using the tetraparental conjugative mating, as described by Heil et al. (2012).

Cultures of the donor strain *E. coli* DH5α (pJH111), the helper strain *E. coli* DH5α (pRK600), the integrase helper strain *E. coli* DH5α (pJC2) and the receptor strain *A. tumefaciens* C58 were prepared by inoculating a single colony in 5 ml of LB medium, with the correct antibiotic of each strain. After the bacterial growth, centrifuge 1.5 ml of each culture in a microtube at 19000x g for 2 min, the pellet twice washed in 0.8 % NaCl and then the pellet resuspended in 100 µL of sterile 0.85 % NaCl.

For the mating, 50 µL of each culture was mixed in a microtube and the total volume plated in LB. For control, 10 µL of each culture was isolated plated in LB. The plates were incubated at 30°C for 24 hours. After the incubation, the mating plates was resuspended in 1 ml of sterile 0.85 % NaCl, passed to microtube and centrifuged at 19000x g for 2 min. The pellet was washed twice in 1 ml of sterile 0.8 % NaCl. The cells were resuspended in 1 ml of 0.85 % NaCl and a 10-fold dilution series to 10^{-5} was made and plated 100 µL of each on selective medium amended with Rf, Gm and x-gal. Plates were then incubated 48 hours at 30°C. The same procedure was made with control spots. The selected transformants were maintained in LB with the antibiotics. The transformants confirmation was performed by PCR using the primer TraI_F and LacZ_R. The transformant selected was namely *A. tumefaciens* At11006.

3.2.3. Detection of the gene *aiiA* in RZ2MS9

The gene *aiiA* (GenBank OGY05307) was founded annotated in the genome draft of *Bacillus* sp. RZ2MS9(MJBF01000001.1) (Batista et al., 2016). To confirm your expression, *in vitro* experiments was performed to confirm the AHL degradation ability. In LB plates amended with x-gal, *A. tumefaciens NTL4* (pZLR4) was plated between *P. agglomerans* 33.1 and *Bacillus* sp. RZ2MS9. The *A. tumefaciens NTL4* (pZLR4) was tested between *A. brasilense* strain Ab-v5 and *Bacillus* sp. RZ2MS9. As control, these strains were plated alone, and for the negative control, *A. tumefaciens NTL4* (pZLR4) was plated between *P. agglomerans* 33.1 and *E. coli* DH5α.

The constructed strain *A. tumefaciens* At11006, was used to confirm its ability in the production of AHL blue-target due to *traI::lacZ* translation fusion. At11006 strain was tested against, *Bacillus* sp. RZ2MS9 a QQ positive control and *E. coli* DH5α, were negative control. For the tests, LB plates amended with x-gal were used.

3.2.4. Knockout of *aiiA* in *Bacillus* sp. RZ2MS9

To test the influence of the gene *aiiA* in RZ2MS9 ability to colonize the maize plants, as well test your importance during interaction with other PGPB, as *A. brasilense,* the knockout directed site using a double plasmid system of CRISPR-Cas9 (Jiang et al., 2015) was chosen. For this, the plasmids pCas (Addgene plasmid 62225) and pTargetF (Addgene plasmid 62226) were kindly provided by Professor Welington Luiz de Araújo, ICB-Department of Microbiology, University of São Paulo.

3.2.4.1. Donor DNA construction

The donor DNA used as template for the recombination was constructed by overlap extension PCR, using the primers Up and Dn (Table 2). The first round of PCR reaction was performed using genomic DNA of RZ2MS9 as DNA template and the volumes: 0.75 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTP (10 µM), 2.5 µl of buffer 10X, 0.5 µl of each couple of primers (10 µM) (Up and Dn), 0.3 µl of DNA polymerase (Sigma®), 19 µl of water, for the end volume of 25 µl. The thermal cycling protocol used was: 2 minutes heating step at 94°C. 30 cycles were as follows: 94°C for 30 seconds for denaturing, 50°C for 30 seconds for annealing, and 72°C for 45 seconds for extension. The final extension set was performed at 72°C for 5 minutes.

The second round of PCR was performed using the PCR products of the first round, under the same reaction conditions, using the primers Up-F and Dn-R. To confirm the cassette sequence, the PCR

product was sequenced using the Sanger platform at the Instituto do Genoma Humano (University of São Paulo, São Paulo, Brazil).

Table 2. Primers used in this work to translational fusion construction of the genes *traI* and *lacZ*, and the DNA donor, using overlap extension PCR.

Primer identification	Primer Sequence 5' – 3'	Product size
TraI_F	ATCGCATGCTTTCGCGCCTTGGAACACCGAC	*traI* region – 750pb
TraI_R	TTGAATCATGTCACGCGGCACTCCTGATC	*traI* region – 750pb
LacZ_F	TGCCGCGTGACATGATTCAAGCTTGCGATCCC	*lacZ* gene – 3070pb
LacZ_R	ATCGCATGCTTATTTTTGACACCAGACCAACTGG	*lacZ* gene – 3070pb
Up-F	ATAGTAACGTTATGACAGTAAAGAAACTTTAT	5' of *aiiA* gene - 275 bp
Up-R	AATAATTGAACACTACCCTACACGCTTTAATAT	5' of *aiiA* gene - 275 bp
Dn-F	AGCGTGTAGGGTAGTGTTCAATTATTGTATACG	3' of *aiiA* gene - 275 bp
Dn-R	5'ATAGTAAGCTTCTATATATACTCCGGGAACA3'	3' of *aiiA* gene - 275 bp

3.2.4.2. Construction of sgRNA and pTargetF-*aiiA*

The sequence of 20 nucleotides, the N_{20} with target PAM sequence NGG, was constructed using the *aiiA* gene sequence, being the last 12 bp highly specific to the target gene (Jiang et al., 2015) and the sequences with the lowest number of potential off-target sites, using for this the online platforms CRISPR RGEN Tools Cas-OFFinder vers. 2.4 (http://www.rgenome.net/cas-offinder/) (Bae et al., 2014) and CHOPCHOP (http://chopchop.cbu.uib.no/) (Labun et al., 2018). The sequences were selected following previously established standards: GC content close to 40 % or higher, specificity score closer to 100 %, out-off-frame score ≥ 50 % and low number of off-targets sites.

One sequence of each platform was tested: N_{20}*aiiA*1 (TTCATTGAGACGGAGCAATC), obtained by the RGEN Tools, and the N_{20}*aiiA*2 (TTTGATCATGCAGGAGGAAA), from CHOPCHOP software. In addition to this 20 bp sequence, another 124 bp compose the sgRNA – N_{20} sequence: 5' cohesive termination of *Bam*HI and 3' cohesive terminal *Eco*RI; the promoter sequence pJ23119 and the scaffold sgRNA (or gRNA). The complete sequence of the sgRNA – N_{20} was described in Table 3.

Table 3: sgRNA-N_{20} sequences of *aiiA* gene of *Bacillus* sp. RZ2MS9, used for pTargetF-*aiiA* construction

sgRNA – N_{20}	sgRNA – N_{20} Sequence for *aiiA* gene
SgN_{20}*aiiA* 1	GATCCTTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTTTCATTGAGACGGAGCAATC*GTTTTAGA GCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTG CTTTTTTTG*TTAA
SgN_{20}*aiiA* 2	GATCCTTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTTTTGATCATGCAGGAGGAAA*GTTTTAGA GCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTG CTTTTTTTG*TTAA

Red – *Bam*HI restriction site; Blue - Promoter pJ23119; Bold font – N_{20}; Underlined – gRNA; Orange – *Eco*RI restriction site.

The oligos sgRNA-$N_{20,}$ were constructed with *tracr*RNA and *cr*RNA fused on an only RNA guide, followed of target sequence N_{20} to the *aiiA* gene. Moreover, to facilitate cloning in the pTargetF, was insert also the sequence of promoter pj23119, and two restrictions sites for the enzymes *BamH*I and *Eco*R1. This oligos sequence containing a total of the 144 bp and was synthesized on direction 5' to 3' and reverse complement. The oligos were resuspended in 100 µM of Tris-EDTA buffer solution. The oligos sgRNA-N_{20} was phosphorylated using T4 polynucleotide Kinase (PNK) (NEB) in a reaction with 2 µM of each oligo; 1x buffer T4 ligase buffer (NEB) and 200 $U.ml^{-1}$ of T4 PNK enzyme for 50 µL of reaction volume. The reaction was incubated in heat block at 37°C for 30 minutes. After this period, 2.5 µL of 1M NaCl solution was added to the phosphorylation reaction and incubated at 95°C for 6 minutes, for oligo annealing. Subsequently, the reactions were cooled for 2 hours at room temperature, and then purified with Illustra ™ GFX ™ PCR DNA and Gel Band Purification Kit and diluted 10 times in sterile deionized water.

For pTargetF-*aiiA* plasmid construction, pTargetF was digested with the enzymes *BamH*I and *Eco*RI, following manufacturer's instructions for the double digest. The digestion was purified using illustra™ GFX™ PCR DNA and Gel Band Purification Kit. The ligation between pTargetF and the oligos annealed was performed using the using the T4 DNA ligase (Invitrogen®), following the recommended instructions and incubate at 16°C overnight.

The ligation product was inserted in *E. coli* DH5α by electroporation (Sambrook et al., 1989) and the transformants selected with 90 $\mu g.ml^{-1}$ of Sp. The transformation confirmation was performed by plasmid extraction with QIAprep Spin miniprep kit (Qiagen) and sent to sequencing in Sanger platform using the primer MoCloF (5' AGCGAGGAAGCGGAAGAGCG 3') (Addegene) a specific primer for the pTargetF plasmid. The plasmid pTargetF-*aiiA*1 and pTargetF-*aiiA*2, were chosen to follow the experiments.

3.2.4.3. Transformation of RZ2MS9 with pCas

For the transformation of *Bacillus* sp. RZ2MS9 with pCas plasmid, two concentrations of plasmidial DNA were evaluated: 30 and 100 ng of pCas plasmid. The transformation was performed using the protocol described by Schurter et al. (1989), with modifications (Almeida et al., 2018 - unpublished). Briefly, one single colony of RZ2MS9 was inoculated in 10 ml of LB with 0.1 % of glycine and incubate overnight at 28°C on shaker at 150 rpm. The culture was diluted 100-fold (optical density (OD_{550nm}: 0.01) in 100 ml of LB with glycine 0.1 % and incubated until an OD_{550nm}: 0.2. The cell was obtained by centrifugation and resuspend in 1/40 volume ice-cold electroporation buffer (400 mM sucrose, 1 mM $MgCl_2$, 7 mM phosphate buffer, pH 6.0), twice. The cells were resuspended in 2.5 ml of electroporation buffer and distribute 800 µl aliquots of ice-cold cells into precooled Genepulser cuvettes, 30 ng and 100 ng of plasmid DNA was added and kept for 10 min at 4°C. Electroporation was performed using: 25 µF capacitor and 1.3 kV, in Bio-Rad Genepulser. The cuvettes were maintained another 10 min at 4°C, and then diluted in 1.2 ml LB and incubated for 2 h at 28°C on a rotary shaker at 150 rpm. The transformants were detectable in LB agar plates with 50 $\mu g.ml^{-1}$ of kanamycin, after 24 hours at 28°C.

The transformation confirmation was validated by plasmid extraction using the QIAprep Spin Miniprep kit (Qiagen), following manufacturer's instructions. The miniprep product was used as DNA template for PCR reaction, performed with the primer pCasF (5'CGGTGCCACTTTTTCAAGTT3') and pCasR (5'GTTTGAGACGAGTCGCTTCC3'), using the same conditions for the construction of donor DNA. Thermal cycling protocol was: 5 min at 94°C followed by 35 cycles: 95°C for 30 seconds; 60°C for 30 seconds and 72°C for 30 seconds. The final extension was performed at 72°C for 7 minutes. The amplicons were observed in agarose gel 1.2 %, and the positives transformants were selected for the next steps, using the identification: RZ2MS9::pCas.

3.2.4.4. Knockout of *aiiA* gene in RZ2MS9 through CRISPR-Cas9 system

The selected transformant RZ2MS9::pCas was grown onto Petri dishes containing LB medium supplemented with L-arabinose (10 mM final concentration) to activate λ-Red gene. After, the strain was again electroporated for insertion of the plasmid pTarget-*aiiA*1 and pTargetF-*aiiA*2. The electroporation events were performed using 300 ng of each plasmid, pTargetF-*aiiA*1 and pTargetF-*aiiA*2 separately, with and without, addition of 600 ng of donor DNA, previously constructed, under the same conditions described before. The transformants selection was performed using 50 $\mu g.ml^{-1}$ of Kn and 600 $\mu g.ml^{-1}$ of Sp, onto Petri dishes with LB medium plates plus arabinose, 10 mM final concentration. The plates were incubating for 24 hours at 28°C.

The transformants from the electroporation using the pTargetF-*aiiA*2 and donor DNA were selected to confirm the knockout of *aiiA* gene. The selected transformants were identified as: RZ2MS9Δ*aiiA*1, RZ2MS9Δ*aiiA*2 and RZ2MS9Δ*aiiA*3.The phenotype of these selected transformants were evaluated *in vitro* assay using the QQ biosensor strain At11006. Onto LB medium amended with x-gal, the *A. tumefaciens* At11006 was plated with RZ2MS9 wild-type, as negative control, and with the transformants RZ2MS9Δ*aiiA*1, RZ2MS9Δ*aiiA*2 and RZ2MS9Δ*aiiA*3. To confirm the *aiiA* gene edition, the DNA of these transformants will be sequenced.

3.3. RESULTS

3.3.1. Construction of *A. tumefaciens* At11006

In first step for the construction of the QQ sensor strain was obtained the translational fusion, *traI::lacZ* (Figure 1) and inserted it into pJH110 plasmid, giving rise to pJH111 plasmids (Figure 2). Then, the plasmid was inserted in *E. coli* DH5α, obtaining colonies Kn resistant and blue color in presence of x-gal (Figure 2).

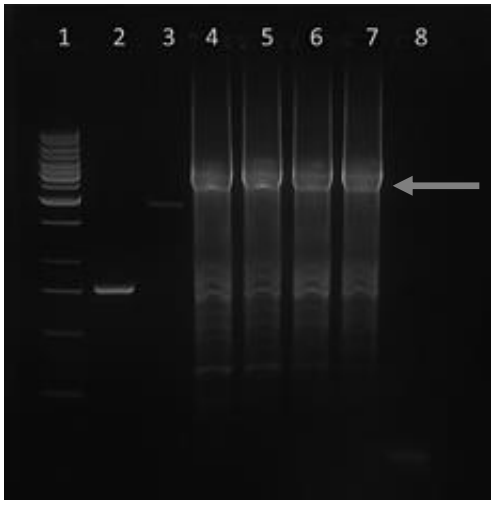

Figure 1. PCR steps for construction *traI::lacZ* translational fusion by overlap extension PCR. Lanes: **(1)** 1KB ladder (Fermentas®); **(2)** *traI* gene (750 bp), obtained by PCR with the primers TraI_F and TraI_R, using the *A. tumefaciens* C58 DNA as template; **(3)** *lacZ* gene (3070 bp), obtained by PCR with the primers LacZ_F and LacZ_R, using the *E. coli* W3110 DNA as template; **(4 -7)** *traI::lacZ* fusion (3820 bp) (arrow indication), obtained from the second round PCR using as template, the PCR reaction of each gene, and the primers TraI_F and LacZ_R; **(8)** PCR reaction control.

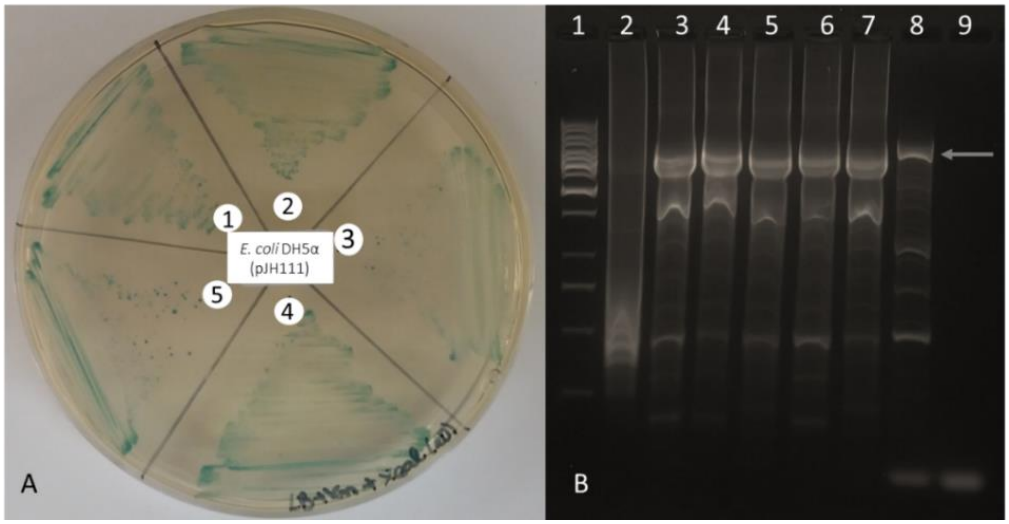

Figure 2. Confirmation of pJH111 construction, inserted in *E. coli* DH5α. (**A**)Transformants *E. coli* DH5α (pJH111) in LB medium with 25 µg.ml^{-1} of Kn and x-gal (20 µg.ml^{-1}). (**B**) Agarose gel 1 % with 1/5 Gel Red (Biotium Inc.), showing the PCR reaction using as DNA template plasmids pJH111-1 **(3)**, pJH111-2 **(4)**, pJH111-3 **(5)**, pJH111-4 **(6)**, pJH111-5 **(7)** extracted from transformants *E. coli* DH5α (pJH111), confirming the *traI::lacZ* gene fusion by the primers TraI_F and LacZ_R. Lanes: **(1)** 1 kb ladder (Fermentas®); **(2)** PCR with pJH110 plasmid as negative control; **(3 – 7)** PCR reaction with pJH111 (pJH110 with *traI::lacZ* gene fusion) derivates as DNA template: pJH111-1; pJH111-2; pJH111-3; pJH111-4; pJH111-5, respectively. **(8)** Fusion *traI::lacZ* as positive control; **(9)** PCR reaction control.

The *A. tumefaciens* At11006 strain was efficiently constructed using the *E. coli* DH5α (pJH111-2) as donor strain of the *traI::lacZ* gene fusion, and the other strains necessary for the tetraparental mating, using for selection x-gal (20 µg.ml^{-1}), Gm (25 µg.ml^{-1}) and Rf (50 µg.ml^{-1}) (Figure 3).

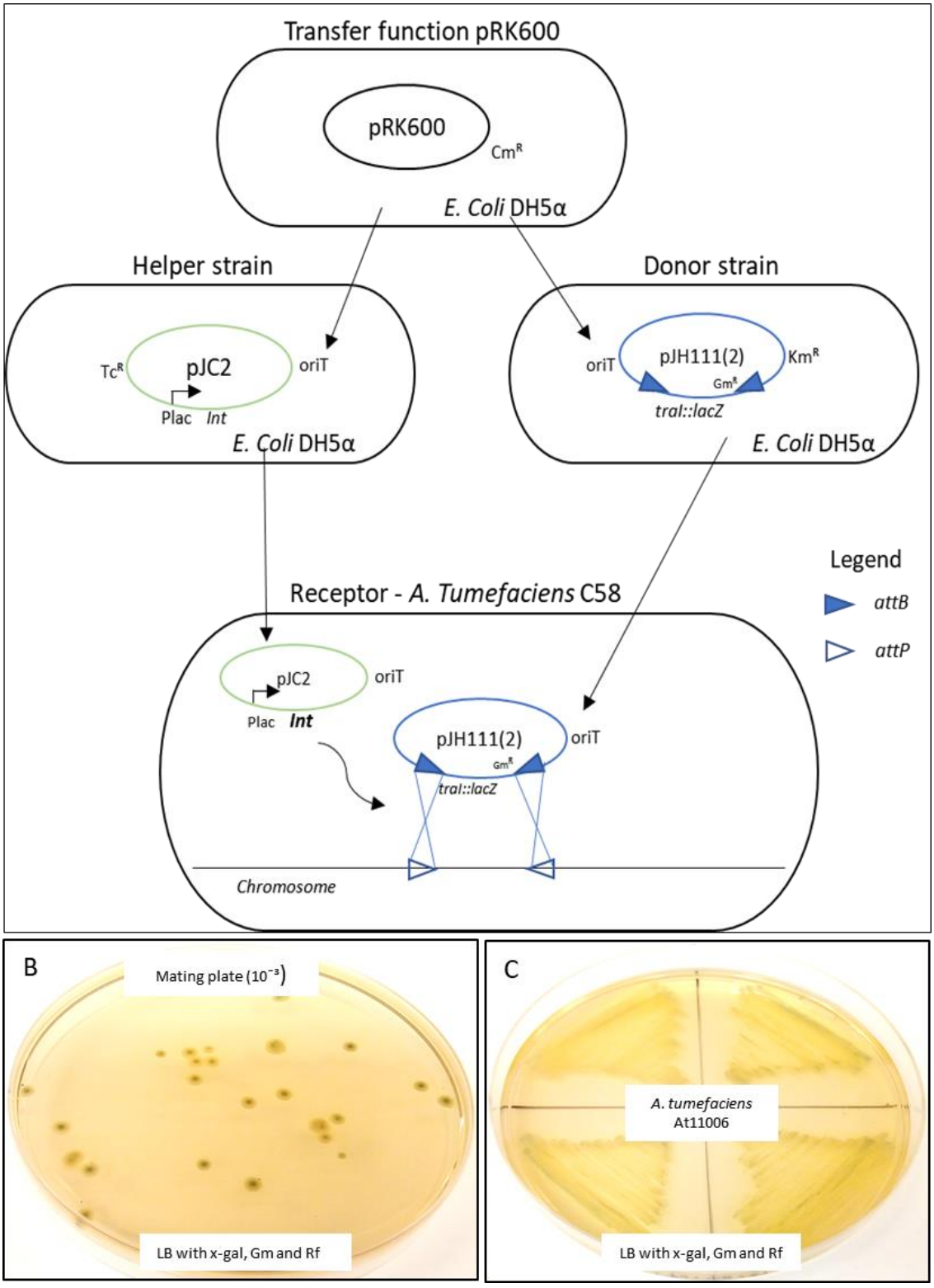

Figure 3. Construction of *A. tumefaciens* At11006. **(A)** Representation of tetraparental mating for construction of At11006 (Adapted from Heil et al., 2012); **(B)** Dilution 10^{-3} performed after the tetraparental mating in selective medium, for selection of *A. tumefaciens* with the *traI::lacZ* fusion; **(C)** *A. tumefaciens* At11006 selected, showing the blue color in selective medium.

3.3.2. Expression of *aiiA* gene in RZ2MS9

The presence of the enzyme *N*-acyl homoserine lactonase (AHL lactonase) in RZ2MS9, that is able to degrade AHL, was confirmed by its capacity to degrade AHL produced by *P. agglomerans* 33.1 and *A. brasiliense* Ab-v5, using *A. tumefacien*s NTL4 as quorum-sensing biosensor strain (Figure 4). *A. tumefacien*s NTL4 in presence of *P. agglomerans* 33.1 (Figure 4A) and *A. brasiliense* (Figure 4D), presented the blue color. As detection control was used: *E. coli* DH5α (Figure 4H), *Bacillus* sp. RZ2MS9 (Figure 4B and 4E) and the biosensor alone (Figure 4I), where in any of these cases, staining was observed.

The production of AHL lactonase was demonstrated by the co-cultivation of *Bacillus* sp. RZ2MS9 with *P. agglomerans* 33.1, in presence of the NTL4 biosensor strain, which no longer presented the blue color (Figure 4C). The same result was observed in the test accomplished with *A. brasilense* Ab-v5 and RZ2MS9, in presence of the NTL4 biosensor strain (Figure 4F). To prove that the color change is not due only to the presence of other bacteria, the *A. tumefacien*s NTL4 was plated between *P. agglomerans* 33.1 and *E. coli* DH5α, being possible to observe the blue coloration of the biosensor, due to AHL produced by *P. agglomerans* (Figure 4G).

*A. tumefacien*s At11006 was tested as QQ biosensor against *Bacillus* sp. RZ2MS9. The result of the interaction between these bacteria only resulted in color intensity decreased of the biosensor strain (Figure 5). As control of the test, the strain DH5α was used (Figure 5B).

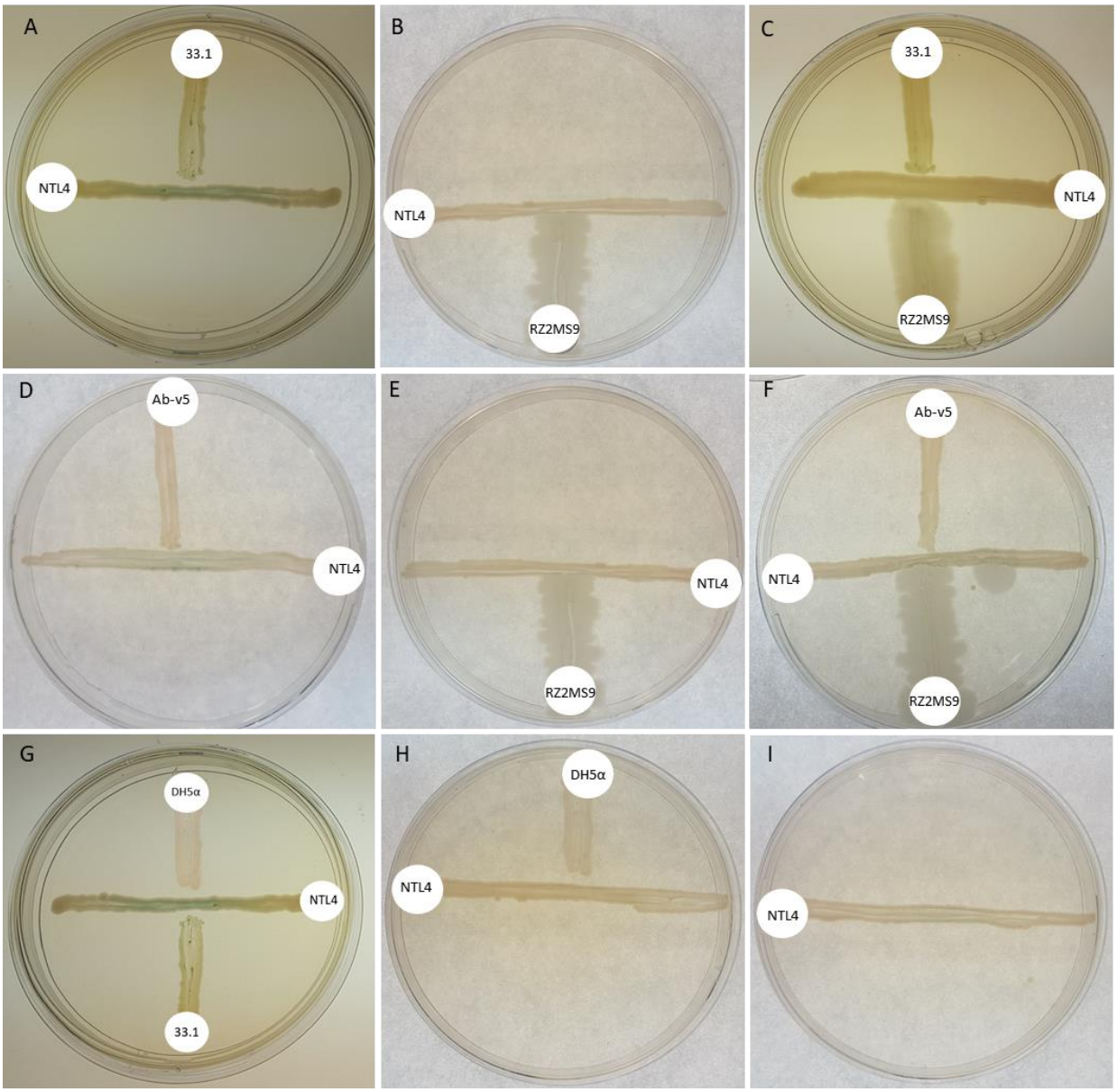

Figure 4: Test with the *A. tumefaciens* NTL4, QS biosensor strain, to confirm the *aiiA* gene expression in *Bacillus* sp. RZ2MS9. All tests were performed in LB plates with 20 µg.ml^{-1} of x-gal, at 28°C for 48 hours. **(A)** *A. tumefaciens* NTL4 with *P. agglomerans* 33.1, where the blue color of the biosensor confirm the AHLs production of 33.1; **(B)** NTL4 with *Bacillus* sp. RZ2MS9, demonstrating the absent of the blue color; **(C)** NTL4 between *P. agglomerans* 33.1 and *Bacillus* sp. RZ2MS9, where the blue color is not observed, demonstrating that AHL lactonase enzyme is active against the AHL of *P. agglomerans;* **(D)** NTL4 with *A. brasilense* Ab-v5, where is possible to observe a slight blue color in NTL4, confirming the production of AHL by Ab-v5; **(E)** NTL4 with *Bacillus* sp. RZ2MS9, demonstrating the absent of the blue color; **(F)** *A. tumefaciens* NTL4 between Ab-v5 and RZ2MS9, when the blue color is not observed, demonstrating the action of AHL lactonase enzyme against AHL of *A. brasilense*; **(G)** Control of experiment using *A. tumefaciens* NTL4 between *E. coli* DH5α and *P. agglomerans,* no demonstrating changes in the blue color of the biosensor strain; **(H)** Experiment control with *A. tumefaciens* NTL4 and *E. coli* DH5α, where the absent of the blue color can be observed; **(I)** Control of experiment using just *A. tumefaciens* NTL4, illustrate lack of coloration in the biosensor strain.

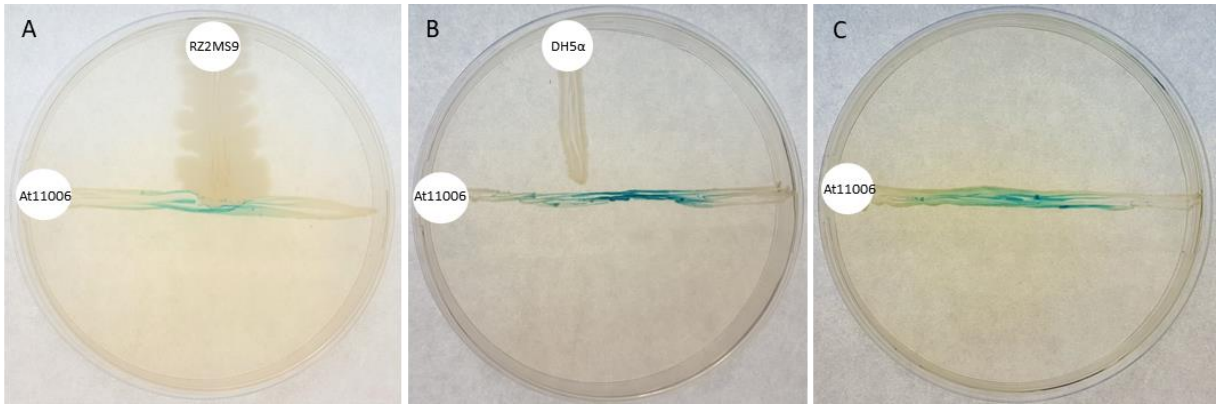

Figure 5: Test with the *A. tumefaciens* At11006, QQ biosensor strain, to test the *aiiA* gene expression in *Bacillus* sp. RZ2MS9, performed in LB plates with x-gal (20 µg.ml^{-1}), incubated at 28°C for 48 hours. **(A)** The biosensor At11006 with RZ2MS9, demonstrating a decrease in the blue color intensity in relation to the control (5C); **(B)** Test control between *E. coli* DH5α and *A. tumefaciens* At11006, where the blue coloration of the biosensor strains can be observed, **(C)** The biosensor strain *A. tumefaciens* At11006, demonstrating your blue coloration.

3.3.3. *aiiA* knockout in *Bacillus* sp. RZ2MS9

The donor DNA was efficiently constructed using the overlap extension PCR technique, deleting a central part of the gene *aiiA*, and your sequence was confirmed by sequencing (Figure 6). The pTargetF-*aiiA* plasmid was efficiently constructed and the presence of sgRNA-N_{20} was confirmed by sequencing. The plasmid pTargetF-*aiiA2* was selected for the next steps.

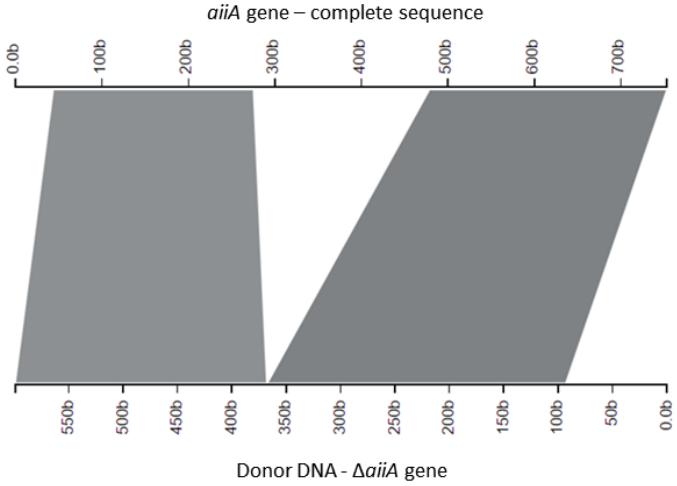

Figure 6. Comparison between the complete sequence of *aiiA* gene and the donor DNA, constructed using overlap PCR. The white region in the graphic represent the central region delete in the donor DNA, present in the complete sequence of *aiiA* gene. Image obtained through the Kablammo program (Wintersinger and Wasmuth, 2015).

The transformation of *Bacillus* sp. RZ2MS9 with pCas plasmid was efficient. The transformation using 30 ng of pCas demonstrated 6.9x10^4 transformants per ng of DNA (Figure 7). The transformation using 100 ng of pCas showed a lower efficiency, 2x10^4 transformants. The selected *Bacillus* sp.

RZ2MS9::pCas, validated by PCR using the primers pCasF and pCasR, was used for a new electroporation process, the next step for the knockout with CRISPR-Cas9 system.

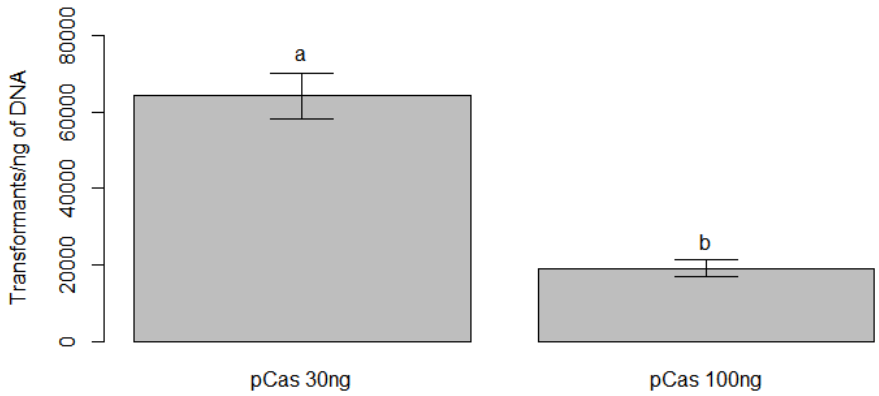

Figure 7. Transformation efficiency of *Bacillus* sp. RZ2MS9 with the plasmid pCas, using the protocol previous described (Almeida et al., 2018 - unpublished). Two concentrations of the plasmid pCas was tested: 30 and 100 ng. The graphic showed the number of transformants colonies 24 hour after transformation in LB plates with 50 $\mu g.ml^{-1}$ of Kn.

Bacillus sp. RZ2MS9::pCas was efficiently transformed with pTargetF-*aiiA2*, in presence and absence of donor DNA. The transformation using RZ2MS9::pCas, pTargetF-*aiiA*2 and the donor DNA showed the highest number of transformants, $6x10^3$ transformants per ng of DNA. The transformation of RZ2MS9::pCas without donor DNA demonstrated an efficiency of $3x10^3$ transformants per ng of DNA (Figure 8).

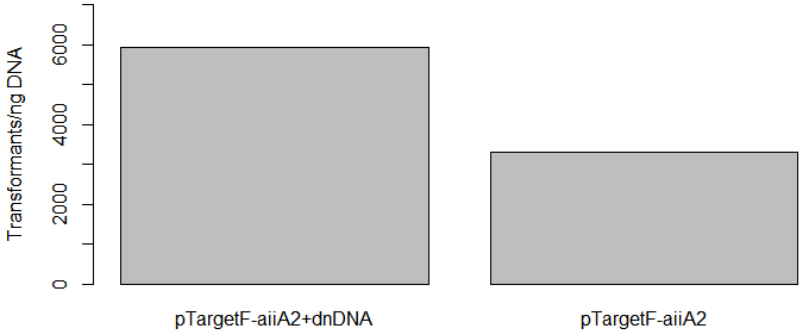

Figure 8. Efficiency of *Bacillus* sp. RZ2MS9::pCas transformation using the plasmid pTargetF-*aiiA*2, in presence and absence of donor DNA (dnDNA). The transformants was obtained, 24 hours after incubation in LB plates with 50 $\mu g.ml^{-1}$ of Kn, 500 $\mu g.ml^{-1}$ of Sp and 10 mM of arabinose.

Among three transformants selected for the plate test against the QQ biosensor, the transformant RZ2MS9Δ*aiiA*2 did not demonstrate difference in the AHL degradation, compared with

the wild-type strain. The transformants RZ2MS9Δ*aiiA*1 and RZ2MS9Δ*aiiA*3 demonstrated a decrease in the AHL degradation (Figure 9).

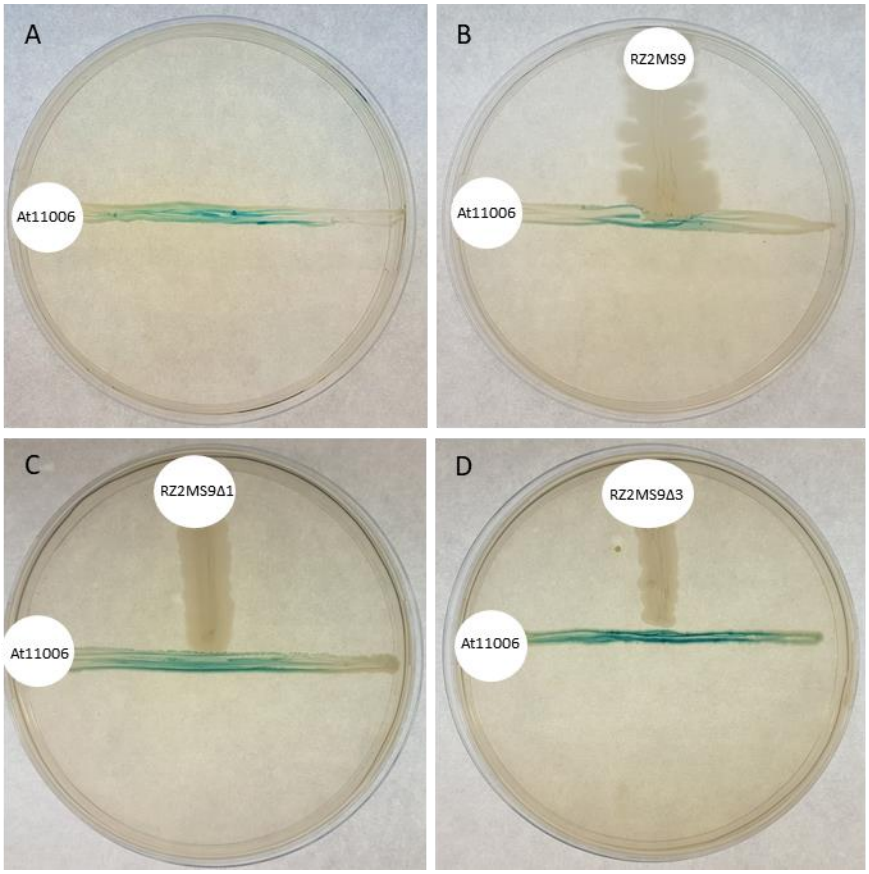

Figure 9. Plate tests performed with the *A. tumefaciens* At11006, QQ biosensor strain **(A),** to test the if the AHL degradation ability demonstrated by RZ2MS9 it is due to the *aiiA* gene. The partial loss of AHL degradation can be observed in the mutant RZ2MS9Δ*aiiA*1 **(C)** and the total ability in the mutant RZ2MS9Δ*aiiA3* **(D)**, in comparison with the RZ2MS9 wild-type **(B)**. The tests were performed in LB plates with x-gal (20 µg.ml^{-1}) under incubation at 28°C for 48 hours.

3.4. DISCUSSION

The ability to degrade AHLs or other lactones gives microorganisms an advantage to survive on a variety of environments as well as out-competing other microbes. The ability to interfere with QS process is considered a natural strategy employed by various microorganisms to out-compete their competitors while in the same ecological niche (Safari et al., 2014). Many microorganisms use QS to regulate the expression of genes such as virulence, antibiotic and/or toxin production (Bassler, 2002), and the ability to degrade the QS signal may interfere in their gene expression enhancing the survival of the microorganism able to degrade these molecules (Safari et al., 2014).

The ability to degrade AHLs or other lactones are not just evolved in intervention of the QS. The lactone-degrading enzymes are probably involved in different biological functions, including degradation of toxic metabolites, in order to avoid their toxic effects (Safari et al., 2014), and others, no extensively described such as possible capacity to promote the plant growth.

Among the three main classes of enzymes able to degrade the AHLs and/or other lactones, the AHL lactonase is the most studied. The AHL lactonase encoded by *aiiA* gene from *Bacillus* sp. 240B1 strain was the first protein identified capable of enzymatic inactivation of *N*-acyl homoserine lactones (Dong et al., 2000). This gene was annotated in the genome draft of *Bacillus* sp. RZ2MS9 (Batista et al., 2016), but your activity had not yet been detected before. This work confirmed the expression of AHL lactonase by *Bacillus* sp. RZ2MS9 through the plate inhibition assay, using the QS biosensor strain *A. tumefaciens* NT4L and the constructed QQ biosensor, *A. tumefaciens* At11006.

The construction of a new QQ biosensor strain using *A. tumefaciens* was chosen as an alternative for detecting a broad range of AHLs (Tang et al., 2013), as *A. tumefaciens* A136 (Fuqua and Winans, 1996) and *A. tumefaciens* NTL4 (pZLR4) (Szenthe and Page, 2003). The strain A136 carry outs the *traI::lacZ* fusion, *traR* in the plasmid pCF218 without Ti plasmid. *A. tumefaciens* NTL4 does not carry out the Ti plasmid, the genes *traG* interrupted by *lacZ* fusion in pZLR4 plasmid, and in same plasmid has the gene *traR*. The new constructed strain, *A. tumefaciens* At11006, has the Ti plasmid and the *traI::lacZ* fusion inserted in its chromosome. The strains A136 and NTL4 do not have natural QS system activated due to absence of Ti plasmid. The same it does not happen with At11006, that present the natural QS pathway production, and the production of AHL-tagged. For this reason, this strain can be used alone as QQ biosensor, once the production of *traI* tagged occurs all the time. So in presence of strains QQ producers, AHL-tagged with *lacZ* and AHL non-tagged will be detected and degraded, generating a reduction of the blue color, in presence of x-gal in the medium.

Usually, the QQ production detection is performed by the use of QS biosensor and chromatography and/or absorbance spectrum (Molina et al., 2003; Kusari et al., 2014; Tang et al., 2013). Thus, the construction of the QQ biosensor *A. tumefaciens* At11006 strain, presents itself as a novelty. At11006 shows the expression of *traI* gene (AHL synthesis) tagged with *lacZ,* as the QS system in this strain is complete (*traR* and Ti plasmid). This strain overproduces AHL, being produced the usual AHL and AHL-tagged. So, because of the fusion with *lacZ,* AHL-tagged present the expression of β-galactosidase, that in x-gal presence showed the blue coloration. This way, At11006 in bioassays with strains QQ enzymes producers, in x-gal presence, will no longer display the blue coloration, or will exhibit the reduction of the intensity of its coloration

One advantage in the use of At11006, in comparison with the other QS strains used in QQ bioassays is the absence of constructed plasmids in the cytoplasm. As the *traI::lacZ* gene fusion was inserted by recombination of *att* site, At11006 just present the Ti plasmid in the cytoplasm. This is an

advantage for functional screening of genomics and/or metagenomics libraries, where the expression of genes in exotic organisms is necessary (Handelsman, 2005) and the plasmids incompatibility (Novick, 1987) that does not allow the use any biosensor strain, is not present in this new strain.

Generally, three main types of QQ identification methods have been described: plate inhibition assay, minimum medium assay, and residual AHL measurement (Tang et al., 2013). The plate inhibition assay is based on agar diffusion of the QS-interfering molecules which would produce a visible inhibition zone in the plates when the co-incubated biosensor produced pigment, or changing the phenotypes (McLean et al., 2004). In the assay in minimum medium the capability of the bacteria utilizes AHLs as sole source of nitrogen, carbon and energy were tested (Chan et al., 2009). The third method is performed by directly measurements of residual AHLs by colorimetry, GC-MS, LC and HPLC36 (Yang et al., 2006).

All of the methods to QQ identification present limitations. For example, the use of assay in minimum medium demonstrates a low efficiency of QQ identification efficiency bacteria because most of the reported bacteria able to produce QQ enzymes is not able to grow in the minimal medium but possess strong AHL-degrading activities (Uroz et al., 2009). The problem of measurement of residual AHL technique is the requirement of particular instruments for the measurement, like GC-MS, LC and/or HPLC, that can limit the application of this identification type of QQ bacteria (Tang et al., 2013). The limitations of the plate inhibition assay are the labor-consuming when testing hundreds of candidates, and a strong QQ activity is necessary to observe a detectable inhibition zone or phenotype change (Tang et al., 2013). And the use of At11006 strain, present this last limitation, as observed in the bioassays performed in this work.

The ability of *Bacillus* sp. RZ2MS9 in degrade AHL produced by *P. agglomerans* 33.1 and *A. brasilense* Ab-v5 was confirmed by the test performed with *A. tumefaciens* NT4L (pZLR4) and confirmed by the performed assays with *A. tumefaciens* At11006. The performed assay with At11006 and RZ2MS9 did not demonstrated the same ability of this strain showed in the other bioassay. Probably the no strong coloration change of At11006 can be result of the large number of AHL molecules produced by At11006, tagged and no tagged by *lacZ*. Furthermore, no strong coloration change of At11006 in presence of RZ2MS9 can be resulted due to a low production of AHL lactonase enzyme by RZ2MS9, just in presence of *A. tumefaciens.*

The *aiiA* gene was first described in *Bacillus* sp. strain 240B1 (Dong et al., 2000). Homologs of *aiiA* were found in other species of the genus as *B. amyloliquefaciens*, *B. subtilis, B. cereus, B. mycoides and B. thuringiensis* (Yin et al., 2010; Dong et al., 2002; Lee et al., 2002; Pan et al., 2008; Uroz et al., 2003). The *aiiA* gene of *Bacillus* sp. RZ2MS9 present 89 % of identity with the *aiiA* gene of *Bacillus* sp. A24 (GenBank AF397400.1), strain that present the ability to degrade AHLs produced by plant pathogens, as *Erwinia carotovora* and *A. tumefaciens*, besides to exhibited broad-spectrum activity by

significantly reducing diseases of potato and tomato caused by these phytopathogenic bacteria (Molina et al., 2003). Similar ability was observed with the AHL lactonase from in *B. amyloliquefaciens,* that expressed *E. coli* BL21 presented the ability to control or decrease of the minor soft rot, caused by *Pectobacterium carotovorum* subsp. carotovorum in carrots (Yin et al., 2010).

To confirm that RZ2MS9 AHL degradation ability is due the activity of AHL lactonase encoded by *aiiA* gene, the knockout of this gene was performed using the newest technique, the CRISPR-Cas9 system. This technique has been used for the most different purposes and allows the genome engineering in prokaryotes and eukaryotes, including *E. coli* (Jiang et al., 2013), *Streptomyces* spp. (Cobb et al., 2014), plants (Shan et al., 2013) and human cell lines (Mali et al., 2013).

The number of works that have been utilized the CRISPR-Cas9 system in bacteria from *Bacillus* genus are not so abundant (Altenbuchner, 2016; Li et al., 2018; Westbrook et al., 2016; Zhang et al., 2016), and none of those used in the works have agronomic applications. So, it is the first work that described the use of the CRISPR-Cas9 system for genome edition of a PGPB.

The first step for the use of CRISPR-Cas9 was the insertion of the Cas 9 enzyme in the strain. The pCas plasmid was efficiently inserted in *Bacillus* sp. RZ2MS9, showing $6x10^4$ transformants per ng of DNA, using 30 ng of plasmid in the transformation process. The second step was the construction of pTargetF-*aiiA*. Using the plasmid pTargetF (Addgene 62226), the only sequence that need to be altered is the N_{20} sequence, that needed to reprogram to change the target site, that was efficiently constructed.

By the action of Cas endonuclease, the CRISPR-Cas9 system generates a double-strand break (DSB), that in eukaryotic cells can be repaired by the erroneous nonhomologous DNA end joining (NHEJ) recombination system, resulting in a mutation at the target site (Gratz et al., 2013). This system of recombination is not present in most of bacteria (Altenbuchner, 2016), and the double-strand break can lead to bacterial death. For this reason, the donor DNA was constructed by PCR overlap and utilized as homologous template for genome edition of RZ2MS9. However, the transformation of RZ2MS9 was also performed without the donor DNA, and transformants was obtained, demonstrating that RZ2MS9 has a nonhomologous recombination system. The presence of this recombination system was also observed by Altenbuchner (2016). The authors, that used the CRISPR-Cas9 system in *Bacillus subtilis* strain, without donor DNA.

The transformation efficiency of RZ2MS9::pCas with pTargetF-*aiiA*2 and the donor DNA was the double of the obtained in the transformation without the donor DNA, $6x10^3$ and $3x10^3$ transformants per ng of DNA, respectively. Although, the use of donor DNA for RZ2MS9 strain transformation with CRISPR-Cas9 system is shown to be more efficient. A low transformation efficiency of *E. coli* using the CRISPR-Cas9 system without donor DNA was observed by Jiang et al. (2015), $7.8x10^{-8}$ CFU/total CFU, a number that can considered resulted from an escape from the death effect of DSB (by Jiang et al., 2015).

Zhang et al. (2016), using the plasmid pHYcas9d and the donor DNA supplied by PCR fragment, did not meet efficiency in the genetic editing of *B. subtilis* ATCC 6051a.

The plate tests using the mutants RZ2MS9Δ*aiiA*1 and RZ2MS9Δ*aiiA*3 against the QQ biosensor At11006 demonstrated an increase in the coloration of the biosensor in presence of the mutants, comparing with the coloration observed in presence of RZ2MS9 wild-type. The color changing observed in the biosensor in presence of mutant RZ2MS9Δ*aiiA*1 was lower than observed in the presence of RZ2MS9 wild-type but is still possible to observe a small change in coloration. The test between At11006 and RZ2MS9Δ*aiiA*3, no coloration change of the biosensor strain was observed, demonstrating the loss of RZ2MS9Δ*aiiA*3 ability to degrade of AHL-tagged produced by At11006. These tests confirm, the efficiency of gene knockout using the CRISPR-Cas9 system in *Bacillus* sp. RZ2MS9, and that the ability in AHL degradation of this strain is due to *aiiA* gene. The gene edition performed in these mutants still needs to be performed by sequencing.

The mutant RZ2MS9Δ*aiiA*3 can be used to understand the influence of the QQ system for the interaction of RZ2MS9 and plants, even as study the influence of this system in the interaction of this *Bacillus* strain with others PGPB. The mutant RZ2MS9Δ*aiiA*3 can be used to better understand the interaction between this strain and *A. brasilense* Ab-v5 during maize colonization. Previous studies demonstrated that co-inoculation of RZ2MS9 and Ab-v5 in maize demonstrated better results of plant growth promotion, when compared with the mono-inoculated strains. Despite this, by the monitoring of co-inoculation of these, the interaction pattern of Ab-v5 with maize was change in presence of RZ2MS9 (Almeida et al., 2018 – unpublished), this way, the mutant RZ2MS9Δ*aiiA*3 can be used to verify the influence of the QQ system of *Bacillus* sp. RZ2MS9 during this interaction with maize.

This study opens the possibility to better understand the role of QQ system in the interaction among PGPB and plants, that can be a way to better the understand the cross-talking communication of PGPB and plants.

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