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Molecular mechanisms involved in the bacterial talking and maize growth  
promotion

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Dissertation presented to obtain the degree of Master in  
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## 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, incluindo 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; *Azospirillum 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, including 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; *Azospirillum 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 which 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 of 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 secrete 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.

## 2. CONCLUSIONS

The eletrotransformation of *Bacillus* sp. RZ2MS9 using the integrative plasmid pNKGFP (Ferreira et al., 2008) was performed efficiently, turning possible monitoring the PGPB RZ2MS9-GFP tagged (RZ2MS9::pNKGFP6) during maize interaction. By fluorescence microscopy (FM) and qPCR, RZ2MS9::pNKGFP6 strain was observed colonizing maize plants, demonstrating an endophytic behavior, being able to penetrate plant roots and migrated through xylem to shoot.

The monitoring of RZ2MS9::pNKGFP6 was also performed during maize colonization under co-inoculation with *Azospirillum. brasilense* Ab-v5, previous tagged with dsRed (Ab-v5::pWM1013) (Tschoeke, 2016). This interaction was also evaluated by qPCR and FM observation, demonstrating that *Bacillus* sp. RZ2MS9::pNKGFP6 was able to co-colonize the same niches previously described as colonized by Ab-v5::pWM1013 in maize (Tschoeke, 2016), however the Ab-v5::pWM1013 was not detected colonizing the maize tissues. 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. At the same time, the better results obtained in plant promotion assay in the co-inoculated plants, demonstrates the occurrence of synergistic interaction between these bacteria and maize. At first time, we clearly demonstrated that RZ2MS9 and Ab-v5 maize co-inoculation presented better results than single inoculation with RZ2MS9, corroborating with previous studies that demonstrated the synergistic effects of co-inoculation in the plant development.

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). In this work, we demonstrated by plates assays that *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. The production of this enzyme can be directed related with the results observed in the interaction of Ab-v5::pWM1013 and RZ2MS9::pNKGFP during maize colonization.

With the aim of make easier the detection of strains able to produce QQ molecules, this study developed a new QQ biosensor strain, the *Agrobacterium tumefaciens* At11006. This biosensor presents the natural QS pathway production, and the expression of *tral* gene (AHL synthesis) tagged with *lacZ*, resulting in a strain that overproduces AHL, being produced the usual AHL and AHL-tagged. So, because of the fusion with *lacZ*, AHL-tagged present the expression of  $\beta$ -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. This biosensor was used to confirm the ability of *Bacillus* RZ2MS9 as AHL-lactonase producer, and the results validated using the quorum sensing biosensor *A. tumefaciens* NT4L (pZLR4) (Szenthe and Page, 2003).

The ability of RZ2MS9 in AHL degradation due the activity of AHL lactonase encoded by *aiiA* gene was confirmed by the knockout of this gene using the technique CRISPR-Cas9 system, obtaining defectives mutants for lactonase production, confirmed by plates assays using the biosensor At11006. The defectives mutants obtained 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.

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. 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, thereby resulting an increase in the use of biofertilizers.

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