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

New insights into Plant Growth Promoting Rhizobacterium *Bacillus thuringiensis* RZ2MS9 biology: entomopathogenic activity and molecular interaction *with Zea mays* L.

Daniel Prezotto Longatto

Thesis presented to obtain the degree of Doctor in Science. Area: Genetics and Plant Breeding

Piracicaba
2020

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

Advisor:
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Tese (Doutorado) - - USP / Escola Superior de Agricultura “Luiz de Queiroz”.

1. Bioinseticida 2. Saúde vegetal 3. Proteínas efetoras 4. Força de dreno 5. Gene miaA I. Título

"1. Naquele dia, saiu Jesus e sentou-se à beira do lago. 2. Acercou-se dele, porém, uma tal multidão, que precisou entrar numa barca. Nela se assentou, enquanto a multidão ficava à margem. 3. E seus discursos foram uma série de parábolas. 4. Disse ele: “Um semeador saiu a semear. E, semeando, parte da semente caiu ao longo do caminho; os pássaros vieram e a comeram. 5. Outra parte caiu em solo pedregoso, onde não havia muita terra, e nasceu logo, porque a terra era pouco profunda. 6. Logo, porém, que o sol nasceu, queimou-se, por falta de raízes. 7. Outras sementes caíram entre os espinhos: os espinhos cresceram e as sufocaram. 8. Outras, enfim, caíram em terra boa: deram frutos, cem por um, sessenta por um, trinta por um. 9. Aquele que tem ouvidos, ouça”.

Mateus, 13:1-9

A DEUS, Santo Antonio, São Bento e Nossa Senhora
Aos meus pais, Antonio Jorge e Eliana,
À minha irmã Carolina, cunhado Ricardo e à bela sobrinha Alice
Com muito carinho dedico este trabalho
Muito obrigado por cuidarem com tanto carinho desta e outras tantas sementes
E por me ajudarem a tornar realidade

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RESUMO

Novos *insights* na biologia da rizobactéria promotora do crescimento em plantas *Bacillus thuringiensis* RZ2MS9: atividade entomopatogênica e interação molecular com *Zea mays* L.

O uso de bioinoculantes nos campos pode aumentar a produtividade final das culturas agrícolas, pela melhoria de uma infinidade de processos nos hospedeiros, incluindo defesa contra pragas e doenças e melhor acesso aos recursos e nutrientes do solo. No entanto, a maioria dos estudos da relação das plantas com microroganismos benéficos se concentrou em bactérias envolvidas na nodulação em Fabaceae, apesar do grande potencial de inoculantes de vida livre. Assim, este trabalho avançou no entendimento da relação positiva entre a Rizobactéria Promotora do Crescimento em Plantas (RPCP) de vida-livre *Bacillus* sp. RZ2MS9 em uma abordagem multidisciplinar. No primeiro capítulo, fornecemos a primeira descrição do potencial entomopatogênico do RZ2MS9, o que corroborou sua classificação como *Bacillus thuringiensis*. Resumidamente, as proteínas de cristal inseticida cubóide e esférica RZ2MS9 detectadas induziram taxas de mortalidade em larvas de *Diatraea saccharalis*, *Helicoverpa armigera*, *Agrotis ipsilon* e *Anthonomus grandis* de forma semelhante ao bioinseticida comercial DiPel® (*Bacillus thuringiensis* serovar *kurstaki* HD1) em ensaios *in vitro*. Adicionalmente, uma região com 67% de identidade em relação à proteína Cry1B de *Bacillus thuringiensis* patogênica para insetos de ordens de Lepidoptera e Coleoptera plasmídeo foi sequenciada a partir de plasmídio de RZ2MS9. No segundo capítulo, foi feita a primeira identificação *in silico* e perfil transcricional de doze proteínas candidatas efetoras que codificam genes de uma PGPR de vida livre, *Bacillus thuringiensis* RZ2MS9, durante a interação com o hospedeiro milho em um sistema gnotobiótico. As sequencias codantes dos genes efetores candidatos de RZ2MS9 abrigaram três motivos exclusivos MEME, e tiveram localização subcelular predita pelo LocTree3: citoplasma do hospedeiro (54,5%), apoplasto (27,3%), cloroplasto (9,1%) e retículo endoplasmático (9,1%). Seis genes efetores candidatos de RZ2MS9 foram associados a ilhas genômicas putativas. No geral, 45% dos transcritos de genes efetores candidatos foram expressos 12 e/ou 120 horas após a inoculação (h.a.i.), OGY05372.1 e OGY05572.1 em ambos, corroborando a eficiencia do *pipeline* utilizado e fornecendo alvos para estudos futuros. No terceiro capítulo, a interação benéfica milho-RZ2MS9 foi avaliada considerando o perfil transcricional dos genes do hospedeiro e indicadores de promoção do crescimento das plantas, como matéria seca, açúcares solúveis e clorofila. RZ2MS9 modulou a expressão de diferentes genes do milho nas folhas e raízes, em relação ao controle, favorecendo maior força de dreno nas raízes e crescimento no estádio V2 em plantas cultivadas em casa de vegetação. A repressão da expressão do gene que codifica a ciszeatina-transglicosidase em raízes de plantas bacterizadas sugeriu menor inativação da citocinina zeatina, ligada à produção de clorofila. A expressão de *lox, pr1* e de beta-glucosidase *bglu*60.1 em folhas de plantas bacterianas sugeriu a ativação da defesa devido ao reconhecimento do RZ2MS9 pelo hospedeiro. Os transcritos do gene RZ2MS9 *miaA*, um marcador genético da produção de citocinina microbiana, foram detectados nas folhas e raízes de plântulas de milho bacterizadas. Maior conteúdo de clorofila foi observado em plântulas de milho bacterizadas cultivadas em casa de vegetação, sugerindo interferência microbiana no equilíbrio hormonal do hospedeiro por meio de um mecanismo a ser estudado em que a liberação no hospedeiro de formas conjugadas de hormônios nas raízes e folhas e

a produção direta de citocinina pela bactéria podem participar. Os genes de milho estudados com comportamento diferencial durante interação RZ2MS9 podem contribuir para estudos adicionais em outros sistemas de milho-PGPR.

Palavras-chave: Bioinseticida, Saúde vegetal, Proteínas efetoras, Força de dreno, Gene *miaA*

ABSTRACT

New insights into Plant Growth Promoting Rhizobacterium *Bacillus thuringiensis* RZ2MS9 biology: entomopathogenic activity and molecular interaction *with Zea mays* L.

The use of bioinoculants into the fields can increase crop final productivity by enhancement of a myriad of host processes, including defense against pests and diseases and improved acess to soil resources and nutrients. Yet, the majority of plant-benefical microrganisms relationship studies focused on bacteria involved in nodulation in Fabaceae despite of higher potential of other inoculants. Hence, this work progressed understanding of benefical relationship between tropical free-living Plant Growth Promoting Rhizobacterium (PGPR) *Bacillus sp.* RZ2MS9 and plants in a multidisciplinary way. In the first chapter, we provided the first description of RZ2MS9 entomopathogenic potential corroborating its classification as a *Bacillus thuringiensis* strain. Briefly, the detected cuboid and spherical RZ2MS9 Insecticidal Crystal Proteins (ICP) killed *Diatraea saccharalis, Helicoverpa armigera, Agrotis ipsilon and Anthonomus grandis* larvae similarly to commercial bioinsecticide DiPel® (*Bacillus thuringiensis* serovar *kurstaki* HD1) in rearing essays. Additionally, a region with 67% identity to the *Bacillus thuringiensis* Cry1B protein pathogenic against insects of orders Lepidoptera and Coleoptera plasmid was sequenced from RZ2MS9 plasmid. In the second chapter, the *in silico* identification and transcriptional profile of twelve candidate effector proteins coding genes from *Bacillus thuringiensis* RZ2MS9 was performed during interaction with maize crop host in a gnotobiotic system. RZ2MS9 candidate effector genes coded sequences harbored three MEME exclusive motifs, and were predicted to target host cytoplasm (54.5%), apoplast (27.3%), chloroplast (9.1%) and endoplasmic reticulum (9.1%) according to LocTree3. Six RZ2MS9 candidate effector genes were associated to putative genomic islands. Overall, 45% of candidate effector genes transcripts were up-expressed at 12 and/or 120 hours post-inoculation (h.p.i.), OGY05372.1 and OGY05572.1 at both times, corroborating the efficiency of pipelines and providing runners for future studies of crop host interaction. In the third chapter, maize-RZ2MS9 benefic interaction was evaluated considering transcriptional profile of maize genes and plant growth promotion indicators such as dry matter, soluble sugars and chlorophyll. RZ2MS9 modulated the expression of different genes in leaves and roots, comparing to control, favoring roots higher sink strength and growth at V2 stage under greenhouse conditions. Down-expression of ciszeatin-transglicosidase in bacterized roots suggested reduced inactivation of cytokinin zeatin, related to chlorophyll production. Up-expression of *lox, pr1*, and beta-glucosidase *bglu*60.1 in leaves of bacterized plants suggested defense activation due to host recognition of RZ2MS9. Transcripts of RZ2MS9 gene *miaA,* a genetic marker of microbial cytokinin production, were detected in leaves and roots of bacterized maize seedlings. Higher chlorophyll content observed in bacterized maize seedlings cultivated in greenhouse suggestted microbial interference into host hormonal balance through a mechanism to be further studied in which host release of hormone bound forms in roots and leaves, and direct production of cytokinin by the bacterium might participate. Maize genes studied with differential expression under RZ2MS9 interaction might contribute to further studies in other maize-PGPR systems.

Keywords: Bioinsecticide, Plant health, Effector proteins, Sink strength, *miaA* gene

1. INTRODUCTION

From immemorial times, agriculture practices remain important to the establishment, endurance and development of modern human societies mainly due to plant supplied services that include food, fiber, biomass, pastures, biofuels and medicines (Nolan & Lenski, 2006; Jez et al., 2016). Since then, the retrieval of higher agricultural yield was obtained mainly by favoring plant growth and development processes with better usage of natural resources (Dey et al., 2014). As crops are surrounded by a complex network of biological interaction, the success of production depends on a benefic interaction with biotic agents (Bravo et al., 2011; García-Fraile et al., 2015; Jez et al., 2016).

The inoculation of Plant Growth Promoting Rhizobacteria (PGPR) may be a sustainable alternative to improve the agricultural productivity due to the microbial interference into host physiological processes, and conversely by increasing plant health through biocontrol of pests and phytopathogens (Azevedo et al, 2000; Hungria et al. 2010; Arruda et al., 2013; Jha et al., 2013; Dey et al., 2014). In this sense, bioinoculants made with PGPR strains capable to benefit the crop host in more than one of decribed processes are preferred (Sansinenea, 2012; García-Fraile et al., 2015).

In a previous study of Laboratory of Microorganisms Genetics (Department of Genetics of ESALQ-USP), several rhizobacteria associated with the guarana plant *Paullinia cupanea* were isolated and selected according to their plant growth promotion ability. Among them, *Bacillus* sp. RZ2MS9 was able to fix nitrogen, produce phytormone auxin and siderophores (Batista et al., 2018). Quantitatively, RZ2MS9 bacterized maize seedlings collected 30 and 60 days after germination (d.a.g.) under greenhouse condions showed, respectively, increases of 35.5% and 39.4% in shoot height, 142.6% and 235.9% increase in shoot dry weight, and 75.4% and 247.8% increase of root system dry weight in relation to Control plants (Batista et al., 2018). Almeida (2018) employed Fluorescence Optical Microscopy (MOF) techniques to monitor the colonization of RZ2MS9::*gfp* in maize. RZ2MS9::*gfp* was found in the stem cylinder where a vascular vessel may be inserted suggesting that the bacterium is capable of systemically colonizing the plant, a desired trait for a PGPR.

Thus, the present work contributed to the comprehension of tropical multi-trait *Bacillus* sp. RZ2MS9 plant growth promotion abilities in a multidisciplinary approach.

In the first chapter, RZ2MS9 had its entomological potential evaluated for the first time corroborating previous RZ2MS9 phylogenetic grouping in the same clade of *Bacillus thuringiensis* strains (Batista, 2017). We observed the production of cuboid and spherical Inseticidal Cristal Proteins (ICP) *in vitro* by RZ2MS9. We confirmed the presence of an ICP coding gene in a plasmid. Finally, we demonstrated that RZ2MS9 was capable to kill Lepidopteran and Coleopteran larvae. Thus, this is the first time RZ2MS9 is referred as a *Bacillus thuringiensis* strain.

In the second chapter, we performed the first screening study of effector candidate genes from RZ2MS9. Initially, a specific pipeline allowed the *in silico* prediction of RZ2MS9 putative secreted proteins, that had their subcellular localization predicted and genomic location investigated. Specific primers were designed to evaluate quantitatively the expression of candidate coding genes by Reverse Transcription Polymerase Chain Reaction quantitative real time (RT-qPCR) reactions performed with maize seedlings harvested 1, 12, and 120 h.p.i. with RZ2MS9 in a gnotobiotic system. Among the twelve effector candidates six were located inside putative genomic islands. The candidates OGY05372.1 and OGY05572.1 were significantly up-expressed at 12 and 120 h.p.i. suggesting the contribution of these two putative cytoplasmic effectors to maize-RZ2MS9 interaction.

In the third chapter, we contributed to the comprehension of RZ2MS9-maize benefic interaction evaluating changes in crop host transcriptional profile during plant-growth promotion at early interaction. Remarkably, the transcriptional profile of analyzed genes under controlled *in vitro* axenic and gnotobiotic growth conditions highly reproduced the profile obtained using maize seedlings cultivated under greenhouse conditions, which validated the proposed *in vitro* cultivation system for further studies. Comparing to control plants, RZ2MS9 bacterization changed the expression of different genes in leaves and roots, which favored higher sink strength and growth in roots at V2 stage considering higher fresh and dry matters and soluble sugars content. The phytostimulatory effect of RZ2MS9 in maize roots was evaluated according to changes in host transcriptional profile which included up-expression of sink strength marker gene *susy*, which coded sucrose synthase, and auxin-responsive gene *iaa14,* related to enchanced auxin activity than observed in roots of control plants. The higher auxin activity is consistent to greater adventitious roots proliferation observed in RZ2MS9 treatments. In maize seedlings cultivated under greenhouse conditions, down-expression of ciszeatin-transglicosidase in bacterized roots

suggested reduced inactivation of cytokinin zeatin in the roots. Up-expression of *lox, pr1*, *bglu*60.1 in leaves of bacterized plants suggested defense activation due to host recognition of RZ2MS9 and microbial interference into host hormonal balance. Higher chlorophyll a and b levels were quantified in bacterized maize plants cultivated under greenhouse conditions. The expression of RZ2MS9 *miaA,* a genetic marker of microbial cytokinin production, was detected in leaves and roots of bacterized plants cultivated *in vitro* and under greenhouse conditions and also during RZ2MS9 growth in a minimal broth employed for cytokinin production. Overall, considering maize genes and parameters evaluated, RZ2MS9 interfered in host metabolism, increased root sink strength, and chlorophyll content increase might be related to a mechanism yet to be further investigated in which release of host hormone conjugates and microbial cytokinins might contribute.

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2. UNRAVELLING ENTOMOPATHOGENIC POTENTIAL OF TROPICAL MULTI-TRAIT PLANT GROWTH PROMOTING RHIZOBACTERIUM *Bacillus* sp. RZ2MS9

ABSTRACT

Plant Growth Promoting Rhizobacteria (PGPR) inoculation has been emerging as an eco-friendly alternative to sustain agricultural yield through host physiological processes improvement and by microbial biocontrol of pests and phytopathogens. According to previous phylogenetic study *Bacillus sp.* RZ2MS9, a tropical multi-trait PGPR with significant maize and soybeans growth promotion, was grouped in clade along with commercial *Bacillus thuringiensis (BT)* strains used as bioinsecticides. In this chapter, *Bacillus sp*. RZ2MS9 entomopathogenic potential was described for the first time using commercial *Bacillus thuringiensis* serovar *kurstaki* HD1 (DiPel®) as positive control. Overall, RZ2MS9 and HD1 sporulated cultures were screened for Insecticidal Crystal Proteins (ICP) production under carbolic-acid and basic fuchsine staining and lethality toward pest insect larvae from Lepidoptera and Coleoptera were accessed. Moreover, a plasmid was detected in RZ2MS9 and the presence of coding ICP genes was proved by PCR performed with RZ2MS9 and HD1 plasmids. RZ2MS9 ICPs were cuboid and spherical. The larvae mortalities rates obtained by RZ2MS9 in artificial diet rearing essays were of 90% for sugarcane borer (*Diatraea saccharalis* F.) 92.5% for old-world cotton bollworm (*Helicoverpa armigera* Hübner) and 30% for *Agrotis ipsilon* larvae. The RZ2MS9 treatment induced severe wing deformities in 40% of *Spodoptera frugiperda* moths statistically different from 2.5% and 5% of HD1 and Control treatments, respectively. 87.5% of *Anthonomus grandis* mortality rate larvae obtained by RZ2MS9 treatment was similar to positive control. RZ2MS9 plasmid region amplified with degenerated *cry*1 family set of *primers* showed 67% of identity to *Bacillus thuringiensis* protein Cry1B pathogenic to insects of Lepidoptera and Coleoptera orders, corroborating the artificial diet essays and optical microscopy results. Other entomopathogenic traits, such as presence of chitinolitic coding genes were found in RZ2MS9 *draft* genome and verified by plate essays. Moreover, entomopathogenic ability revealed in this work aggregated to RZ2MS9 great crop growth enhancing potential as new bioinsecticide from Brazilian biodiversity to make plants healthier and more productive.

Keywords: Crystal proteins, Bioinsecticide, Plant health, Crop inoculant, Biological control

2.1. INTRODUCTION

Besides water, soil and climate constraints world crop yield is constantly threatened by weeds, pest and pathogen attacks. Despite integrated pest management adoption, total global agriculture potential loss due to pests attack is 18% to 26% across the crops (Oerke, 2006; Culliney, 2014). Moreover, Brazilian total estimated insect-pest agricultural damages reached 7.7% of major cultures production, which equates to US$ 14.7 billion annual economy loss (Oliveira et al., 2014).

Over the decades, the use of chemicals to pest control has progressed due to their broad-spectrum larvae toxicity (Bravo et al., 2007). However, the insecticides may accumulate in water, soil and food resulting in several diseases and disturbs in animals, humans and insect pollinators (Kouser & Qaim, 2011), as well as the improvement of pest resistance (de Maagd et al., 2001; Bravo et al., 2011). Hence, the search for more specific and ecologically sustainable alternatives to pest control have increased in last decades (Raddadi et al., 2007; García-Hernández et al., 2009; Kouser & Qaim, 2011; García-Fraile et al., 2015). Moreover, cultural and ecologically based biological strategies to pest control provided 7 to 70 folds higher estimated return per dollar invested comparing to chemical control (Culliney et al., 2014). In this context, beneficial microbes previously related as plant growth promoters might be investigated as potential pest control agents. Still, few authors addressed the multi-trait growth promotion vocation of *Bacillus thuringiensis* and related species (Raddadi et al., 2007; 2008; Bravo et al., 2011).

The well-known soil-borne gram-positive bacterium *Bacillus thuringiensis* Berliner (Berliner, 1915) has a remarkable entomopathogenic action against specific insect orders, according to subtypes of a myriad of sporulation-driven synthesized Insecticidal Crystal Proteins (ICPs) coded by cry genes (Navon et al., 1993; Luthy & Wolfersberger, 2000; Bravo et al., 2007). The toxin binding in specific receptors in the epithelial insect midgut creates pores which increase epithelial cells membrane permeability (Luthy & Wolfersberger, 2000). Cells begin to lyse and the feeding activity cease (Schwart & Laprade, 2000). Finally, larvae die of starvation, septicemia or both (Porcar & Juarez-Perez, 2003).

The morphology of *B. thuringiensis* Insecticidal Crystal Protein was addressed by different authors (Höfte & Whiteley 1989; Martins et al., 2008; Silva et al., 2010). Each *B. thuringiensis* strain can harbor more than one crystal type, and some of them are composed of several proteins that have distinct insecticidal activities. The insecticide crystals biosynthesis varied in their levels and spectra of toxicity (Aronson et al., 1986, Bravo et al., 2007). Because most of cry genes are harbored in plasmids (Sansinenea, 2012; Bravo et al., 2011) specific cry genes detection have been successfully performed by PCR using plasmidial DNA templates (Juarez-Pérez et al., 1997; Martinez, Ibarra, & Caballero, 2005; Noguera & Ibarra, 2010) or by plasmid-sequencing (Bravo et al., 2011).

Enzymes may act synergistically with ICPs to enhance entomopathogenic activity, such as chitinases and chitosanases (Wiwat et al., 2000; Arora et al., 2003), that together with chitin deacetylase and glucosaminidase, involve in the decomposition of insect cuticle and peritrophic midgut membrane (Tellam et al., 1999; Kobayashi et al., 2002; Hsu et al., 2012). Other enzymes included ureases (Kappaun et al., 2018), proteases (Fedhila et al., 2002), phospholipase C (Zhang et al., 1993) and hemolysins. Some zinc metalloproteases named immune inhibitor A and B (InhA, inhA2 and InhB) are also released by the bacteria to hydrolyze insect antibacterial proteins (Dalhammar & Steiner, 1984). Recently, bacterial hydrogen cyanide production was also related with larval mortality (Short et al., 2018).

Numerous *Bacillus* spp. strains showed good ability to control caterpillars for over 50 years (*B. thuringiensis* serovar *kurstaki*, *B. thuringiensis* serovar *aizawai*), and more recently mosquito larvae (*B. thuringiensis* serovar *israelensis* and *B. sphaericus*) and beetle larvae (*B. thuringiensis* serovar *tenebrionis*). *B. popilliae* were white grubs pathogens (Lerecluz et al., 1993; Bravo et al., 2007). There are some difficulties with *B. thuringiensis* crop application when target larvae live inside host plants structures such as fruits, poaceae's cartridges and stalks (Downing et al. 2000; Sosa Gómez et al., 2016. The plant inoculation of endophytic microorganisms living in the same insect pests niche (Barboza-Corona et al. 2003) could ease bacterial access to arthropod host (Sansinenea, 2012; Culliney et al., 2014).

In recent studies, our group isolated and evaluated potential plant growth-promoting bacteria from the rhizosphere of guarana plant (*Paullinia cupanea*). Among those strains, *Bacillus* sp. RZ2MS9 *in vitro* was able to fix nitrogen, solubilize inorganic phosphate, and produce auxin and siderophores (Batista et al., 2018). In quantitative terms, maize Altavista cultivar seed inoculation with RZ2MS9 collected 30 and 60 days after germination showed increases of 35.5% and 39.4% at shoot height, respectively, 142.6% and 235.9% increases in aerial part dry weight, and also increase of 75.4% and 247.8% root system dry weight increase comparing to Control treatments (Batista et al., 2018). RZ2MS9 *draft* genome is available at National Center of Biotechnology Information (NCBI) (Batista et al., 2016).

Bacillus spp. are difficult to classify and demand a holistic approach to obtain precise classification. Our group started RZ2MS9 taxonomy studies by performing a robust phylogenetic analysis with sequences of pyruvate carboxylase gene (*pycA*) from other 105 *Bacillus cereus* sensu latu strains sequences. As a result, RZ2MS9 was

grouped in clade along with eight commercial entomopathogenic *Bacillus thuringiensis* strains. Moreover, comparison of functional COG classification of several housekeeping annotated genes of the strains present in the aforementioned clade corroborated physiologic resemblance among them (Batista, 2017). Thus, in this work, we investigated RZ2MS9 entomopathogenic potential from the screening of *in vitro* insecticidal crystalline proteins production*;* presence of ICP coding genes in chromosomal and plasmid DNA by amplification using degenerated primers; detection of entomopathogenic canonical genes in the genome and RZ2MS9 effectiveness to control Lepidoptera and Coleoptera larvae when fed up to them in artificial diets. Altogether, our findings supported the classification of this strain presumably as *B. thuringiensis* as well aggregate to RZ2MS9 already described plant health promotion ability by unfolding its insect biocontrol capabilities and possible use as bioinsecticide.

2.2. MATERIAL AND METHODS

2.2.1. Bacterial strains and culture conditions

Bacillus sp. RZ2MS9 was isolated from guaraná (*Paullinea cupana*) rhizosphere at Maues-AM Brazil (Batista et al., 2018). *Bacillus cereus* strictu sensu BG0269 and *Bacillus thuringiensis* BGJL68 were isolated from mangrove at Bertioga – SP, Brazil (Dr. Joelma Marcon, personal communication). RZ2MS9, BG0269 and BGJL68 belong to Laboratory of Microrganisms Genetics “Prof. João Lúcio de Azevedo”, Department of Genetics, University of São Paulo, Piracicaba-SP, Brazil. Commercial DiPel® (*Bacillus thuringiensis* serovar *kurstaki* HD1) was kindly ceded by professor Dr Italo Delalibera Junior, Pathology and Microbial Control Laboratory, Department of Entomology and Acarology, University of São Paulo, Piracicaba-SP, Brazil. Bacterial colonies were stored in 20% glycerol at -80 °C and routinely cultivated in broth or LB-agar (Sambrook & Russel, 2001). Fresh cultures were started from glycerol stocks for each experiment by plating portions onto in Luria-Bertani (LB) agar and incubated at 28 °C for 24 h.

2.2.2. PCR Cry genes detection

Bacterial total DNA and Plasmidial DNA were used as templates to strain detection of *cry* genes. Bacterial DNA was extracted using DNeasy ® Blood & Tissue Kit (QIAGEN), according to manufacturer protocol. Plasmids were extracted from

RZ2MS9 and HD1 culture cells grown in minimal media supplement with casaminoacids (0,2%) for 20 h by sucrose gradient lysis protocol amended with lysozime (50mg/L) and proteinase K (20mg/L) enzymatic treatments (Reyes-Ramirez, 2008). Plasmidial DNA was analyzed by 0.5 % agarose gel electrophoresis in Tris-Acetic Acid-EDTA buffer (Sambrook & Russell, 2001; Reyes-Ramirez, 2008). Degenerated primers JI(+) and JI(-) were used to detect presence of cry1 coding genes (Juarez-Pérez et al., 1997). Following primers combinations (primer N1 with primer N4; primer N1 with primer N5) were employed to detect novel three-domain cry coding genes (Noguera & Ibarra, 2010) using *Bacillus cereus* strictu sensu BG0269 as a negative control (Table 1).

Table 1 – List of degenerated primers sequences used for detection of cry coding genes in *Bacillus* strains. References were shown.

Primer	5'→ 3' Sequence	Reference
JI(-)	MDATYTCTAKRTCTTGACTA	Juarez-Pérez et al., 1997
JI(+)	TRACRHTDDBDGTATTAGAT	
N1 (F)	TATGCWCAAGCWGCCAATYTWCATYT	Noguera & Ibarra, 2010
N4 (R)	CATAACGTAGWYTTAYCTKAWT	
N5 (R)	GGRATAAATTCAATTYKRTCWA	

Each 50 µL reaction consisted in 250 ng of total *Bacillus* DNA with 2.5 U of *Taq* DNA polymerase (Thermo Scientific), 200 nM each deoxynucleoside triphosphate, 1 mM reverse primer I(-) and 1 mM forward primer and 3 mM MgCl2. Amplification was done in a Applied Biosystems 5000 thermal cycler under the following conditions: 5 min of denaturation at 94°C followed by 25 cycles of amplification with a 1-min denaturation at 94°C, 45 s of annealing at 48°C, and 2 min of extension at 72°C. An extra extension step of 10 min at 72°C was added after completion of the 35 cycles. PCR products were analyzed by 1% agarose gel electrophoresis in 1 X Tris-Acetic Acid-EDTA buffer (Sambrook & Russell, 2001). Bands were excised from the agarose gel and purified using GFX purification gel and band kit (GE Healthcare®) according to supplier conditions. Purified fragments were sequenced at Human Genome Center of University of Sao Paulo, São Paulo, Brazil.

2.2.3. Co-production of spores and parasporal insecticide crystal proteins

Basal Sporulation Broth (BSB) proposed in this work for *Bacillus* RZ2MS9, HD1 and BG0262 sporulation considered minimal nutritional requirements to favor

endospore production as previously described for genus *Bacillus* spp (Guirard & Snell, 1962). BSB contained (g/L): beef peptone (5), peptone (5), NaCl (7.5) amended with glucose (10), yeast extract (3), KH_2PO_4 (2), $MgSO_4$.7H2O (1.5), deionized water (qsp) and pH adjusted to 7.0. Inoculum consisted in addition of log-phase *Bacillus* culture grown in LB broth for 8h at 28ºC to an optical density of 0.2 (600 nm) into basal medium. Inoculated basal medium were then incubated at 37ºC and 150 rpm for 5 days. The full sporulated colonies-ICP mixture was swabbed in glass slides, heat fixed for 20 s and submitted to basic-fuchsine/carbolic acid crystal protein staining accordingly to Zhou et al. (2011) and malachite green staining (Sambrook & Russell, 2001).

2.2.4. *In vitro* evaluation of RZ2MS9 entomopathogenic potential against agricultural insect pests' larvae

In vitro bioassays were performed in order to evaluate RZ2MS9 direct entomopathogenic potential by delivering the mixture of spores and ICPs produced by the bacteria during sporulation into modified artificial diet fed up to different Lepidoptera and Coleoptera agricultural pests. Artificial diets were adapted by exchanging substances toxic to RZ2MS9 or HD1 to allow the survival of these bacteria in the diet.

2.2.5. Artificial insect diet modification

Natural susceptibility of *Bacillus* spp. strains *in vitro* to broadly employed insect diet bacteriostatic agents Tetracycline and formaldehyde (Campos, 2017) motivated artificial insect diet (King & Hartley, 1985; Nalim, 1991) modifications proposed in this work. *Bacillus* survival was evaluated streaking *Bacillus* sp. RZ2MS9 and HD1 fresh cultured cells into LB agar plates (Sambrok & Maniatz, 2001) amended to 0, 50, 100 and 150 mg/L of four antibiotics. The antibiotics used were Tetracycline, Streptomycin, Ampicillin and Penicillin G. The diet adaptations also included Nipagin® reduction to half dosage and absence of formaldehyde according to proposed by Campos (2017).

2.2.6. Insect general rearing conditions

Lepidopteran larvae essays were composed of three treatments with 6 replicates of 10 entomologic glasses each. Coleopteran larvae essays were composed of three treatments with 4 replicates of 12 wells each (made in 24 wells ELISA plates). Each glass or ELISA plate well contained 4-8 mL of proper adapted artificial diet according

to insect species nutritional requirements amended with one of the following solutions: medium BSB (“Broth-control” treatment), 10^8 CFU/mL of RZ2MS9 BSB sporulated cultures for 120h at 37ºC (“RZ2MS9 culture” treatment), 10^8 CFU/mL of *B. thuringiensis* serovar *kurstaki* HD1 BSB sporulated cultures for 120h at 37ºC (“DiPel culture” treatment). Commercial bioinsecticide DiPel® was used as a positive control due to its known ability to control different lepidopterans and other insects (Moar et al., 1990; Wiwat et al., 2000; Arora et al., 2003; Bravo et al., 2011). Each biological sample consisted in one larvae placed in diet surface per glass or ELISA plate well with soft brush. All larvae used in rearing essays were routinely maintained on an artificial diet in an environmentally controlled room under the following rearing conditions: 25 °C, 60 % relative humidity and 14 h photophase at the Laboratory of Insect Biology of the Department of Entomology and Acarology, University of São Paulo, Piracicaba, SP, Brazil (Parra, 1996).

2.2.7. Lepidopteran larvae mortality essays

Bioassays were conducted using *D. saccharalis, H. armigera*, *S. frugiperda* and *Agrotis iplison* neonates larvae emerged from eggs (King & Hartley, 1985; Nalim, 1991). *H. armigera*, *S. frugiperda* and *Agrotis iplison* larvae were fed with the artificial diet modification proposed in this work: addition of penicillin G (100mg/L), half of Nipagin® dosage and absence of formaldehyde. The *D. saccharallis* essay was made using the same conditions described amending streptomycin (50mg/L) and ampicillin (100mg/L). The larvae mortality rate represented the mean percentage of dead larvae for six replicates of ten larvae each for every essay 7 and 21 d.a.i. (*H. armigera*, *S. frugiperda*), 12 and 21 d.a.i. (*D. saccharalis),* 7 and 60 dai (*Agrotis ipsilon*) according to insects biology (Parra et al., 2010; Dinardo-Miranda et al., 2012; Sosa-Gómez et al., 2016).

2.2.8. Coleopteran larvae mortality essays

Bioassays were conducted using *A. grandis* neonates larvae emerged from eggs. *A. grandis* larvae were fed with the artificial diet modification proposed in this work: addition of penicillin G (100mg/L), half of Nipagin® dosage and absence of formaldehyde. Each biological sample consisted in one coleopteran larvae placed in diet surface (ELISA plate well) with soft brush. The larvae mortality rate represented

the mean percentage of dead larvae for four replicates of twelve larvae each evaluated 45 days after inoculation (dai) according to insect biology (Martins et al., 2008).

2.2.9. Additional features related with RZ2MS9 bioinsecticide activity

2.2.9.1. *In silico* search for genomic entomopathogenic traits

Genomic mining in *Bacillus* sp. RZ2MS9 *draft* genome (MJBF00000000) and *Bacillus thuringiensis* serovar *kurstaki* HD1 (JMHW00000000) were made by direct search in NCBI repository of entomocidal characteristics coded by chromosomal genes (Raddhadi et al., 2007; Sansinenea, 2012). The following genes were targeted: ICPs coding genes, urease operon, hydrogen cyanide operon, phospho-lipase C, hemolysins, immune inhibitor A, chitinases and chitosanases.

2.2.9.2. *In vitro* plate essays

RZ2MS9 and HD1 were evaluated for protease and chitinase activities by measurement of halum formed in indicator media containing the respective substrates. Enzymatic index was calculated considering the ratio between average halum diameters over average colony diameter. For each bacterium, six replicates were employed. Proteolitic activity was evaluated using a milk agar medium containing (g/L) powdered milk (10), yeast extract (0.5), ammonium sulfate (0.5), calcium chloride (0.5), potassium phosphate monobasic (0.1), potassium phosphate dibasic (0.1), and agar (18) and pH adjusted to 7.0 ± 0.2 (Akinrinlola, 2018). Chitinase activity was evaluated plating 10 uL drops of *Bacillus* RZ2MS9 and HD1 cultures (10^8 CFU/mL each) in M9 medium with deacetilated colloidal chitin as sole carbon source, plus 0.25g per L of yeast extract (adapted from Sambrook & Russell, 2001) at 28ºC for 5 days. Positive control was chitinolitic *B. thuringiensis* strain JL63 kindly ceded by Dr. Joelma Marcon. Hydrogen cyanide production was evaluated for both *Bacillus* strains using protocol described by Capuccino and Sherman (2004) at 25ºC for 3 days.

2.2.10. Statistical analysis

Data analysis was carried out with the R studio software package “Laercio”. A completely random design was used for all assays. The effects of RZ2MS9 and HD1 spores and ICPs on t larval mortality were determined by counting the number of dead larvae in previously described collecting points (7, 12, 25 and 60 dai, depending on the

insect). The mortality was represented as the mean percentage of dead larvae according to Tukey's test ($p > 0.05$) of six replicates of ten larvae each for lepidopteran essays. For Coleopteran essay four replicates of twelve larvae each were used for each treatment. Solubilization index data from chitolytic and proteolytic plate essays were processed using Duncan's multiple range test.

2.3. RESULTS

2.3.1. *In vitro* optimization of endospores and parasporal crystal proteins production

Maximum sporulation was reached for the strains: RZ2MS9, HD1 (DiPel®) and *B. cereus* strictu sensu BG0269 after 120 h of 37ºC growth in BSB yielding 98% percent of endospores counted in Newbauer chamber (arrows in Figure 1A-1E). Insecticidal Crystal Proteins (ICP) co-production was obtained for RZ2MS9 and DiPel® as revealed by basic-fuchsine carbolic-acid staining (black triangles in figures 1A-1D). As shown in figure 1A-1C *Bacillus sp.* RZ2MS9 produced two different sized ICPs: larger cuboid (Figure 1A left triangle, Figure 1B) and smaller spherical crystals (Figure 1A right triangle, and Figure 1C).

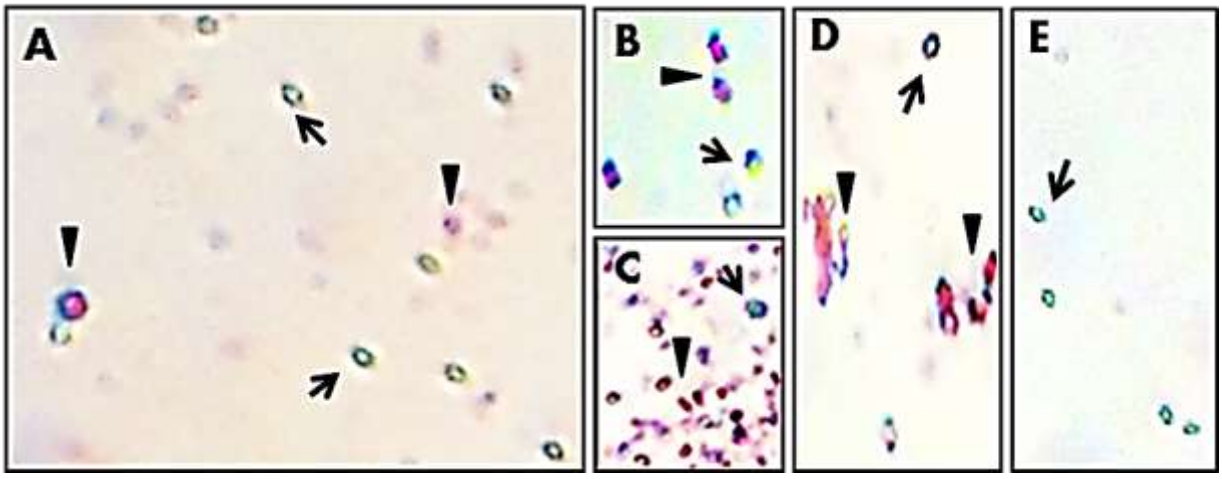

Figure 1: Co-production of endospores and parasporal crystal proteins in *Bacillus* strains: 1A-1C: B. *thuringiensis* RZ2MS9; 1D: *B. thuringiensis* serovar *kurstaki* HD1, 1E: *Bacillus cereus* strictu sensu BG0268. Black arrows spotted malachite green colored endospores. Insecticidal Crystal Proteins morphology variation was also highlighted between *Bacillus* strains under basic-fuchsine carbolic-acid staining (black triangles): RZ2MS9 produced cuboid endospore-sized crystals (Figure 1A left and figure 1B black triangles) and spherical crystals smaller than the endospore (Figure 1A right and figure 1C black triangles). Typical bipyramidal HD1 crystals were spotted in figure 1D (hollow triangle), they usually are bigger than endospores as previously described. Crystal formation was absent for *B. cereus* strictu sensu (1E) using the same culture, sampling and staining procedure.

Bipyramidal crystals were detected in HD1 sporulated culture (Figure 1D). None crystalline formation was detected for the negative control (*B. cereus* strictu sensu BG0268) under the same conditions (Figure 1E).

2.3.2. Plasmids extraction and Cry coding genes detection by PCR

Amplicons range of 0,9 to 1.5 kb (lanes 2 and 3) and 2 kb (lanes 4 and 5) were obtained using degenerated primers I(+) and I(-) respectively using RZ2MS9 and HD1 plasmids. None amplicon was observed using BG0262 (*B. cereus* strictu sensu) Plasmidial DNA as a template (Figure 2). Faint bands were obtained using RZ2MS9 and HD1 total DNA as template, and any was found using BG0262 total DNA as template (data not shown). The RZ2MS9 amplicon (Lanes 2 and 3) showed 67% of homology to *Bacillus thuringiensis* protein Cry1B (N. acess: ABL60921.1). The sequenced HD1 amplicon presented 82% of similarity with *Bacillus thuringiensis* Cry1Ia delta-endotoxin gene (Partial Cds) (N. acess: KJ619662.1). The amplification using two pairs of degenerated primers for three domain cry detection (Noguera & Ibarra, 2010) resulted in 85% of similarity to *Bacillus thuringiensis* protein Cry1Ia delta-endotoxin gene (Partial Cds) (N. acess:KJ619662.1) (Lanes:21 and 22). Fragments amplified from lanes 13, 14 19 and 20 using RZ2MS9 plasmidial DNA template were not similar to any sequence available in NCBI database, suggesting novel cry sequences.

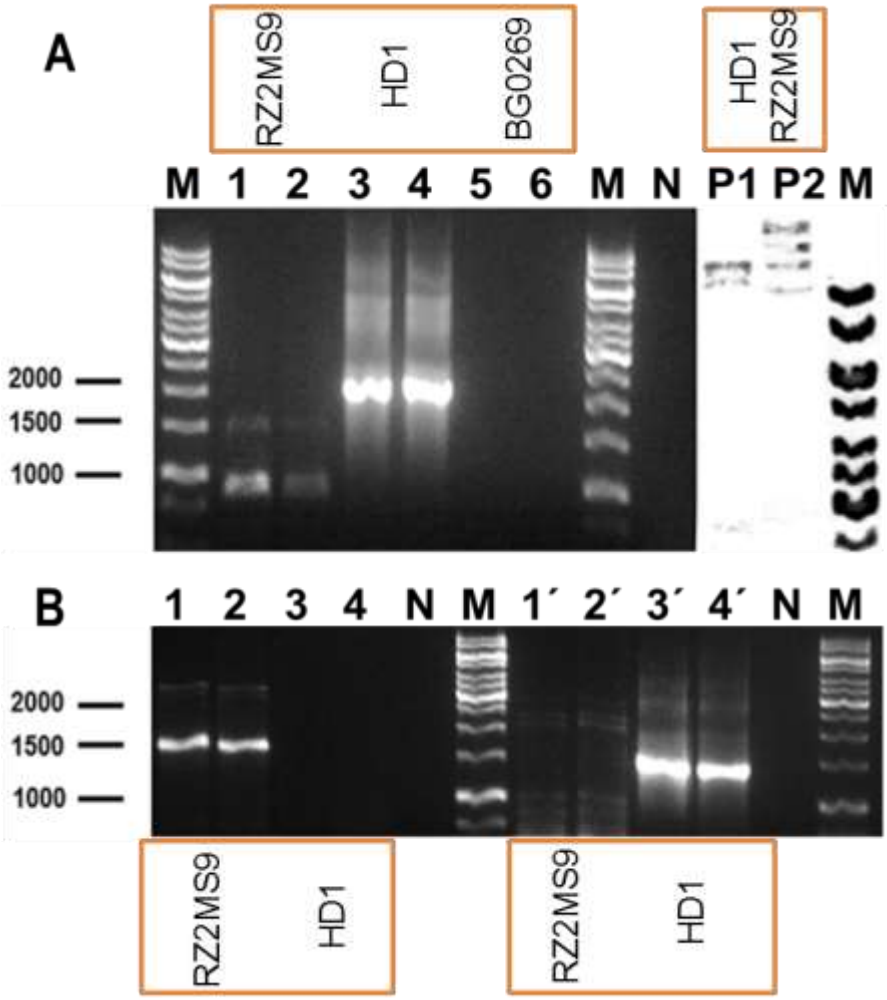

Figure 2: Detection of plasmids in *Bacillus* spp. strains using Plasmidial DNA used as template for *cry* genes detection. A: lane P1: 800ng of HD1 Plasmidial DNA; lane P2: 800ng of RZ2MS9 Plasmidial DNA. PCR reactions performed with cry1 family degenerated primers I(+) and I(-) (RZ2MS9: lanes 1-2; HD1: lanes 3-4; BG0268: lanes 5-6). B: PCR reactions performed with degenerated primers 1 and 4 (RZ2MS9: lanes 1-2; HD1: lanes 3-4) and primers 1 and 5 (RZ2MS9: lanes 1´-2´; HD1: lanes 3´-4´). Lanes M: Molecular weight marker 1Kb DNA Ladder (Thermo Scientific). Lanes N: PCR negative control.

2.3.3. *In vitro* evaluation of RZ2MS9 entomopathogenic potential against agricultural pests' larvae

2.3.3.1. Optimization of artificial insect diet

RZ2MS9 and HD1 were able to grow respectively at 100 and 50 mg/L of streptomycin, 100 and 100mg/L of ampicillin, 100 and 100mg/L of penicillin G.

2.3.3.2. *Diatraea saccharalis* bioassay

The mortality rate of *D. saccharalis* newborn larvae fed with the artificial diet supplemented with RZ2MS9 and HD1 full sporulated cultures (10^8 CFU/mL each) were both significantly higher 72.5% and 92.5%, 12 d.a.i., and 98.5% and 100%, 21 d.a.i. respectively comparing with control (15%, 12 d.a.i. and 17.5%, 21 d.a.i.) according to Tukey's test (5% significance) (Figure 3).

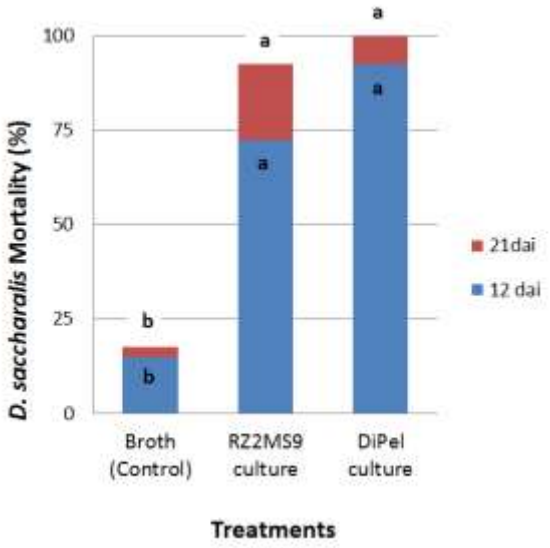

Figure 3. Effects of RZ2MS9 and HD1 sporulated cultures on *Diatraea saccharalis* larvae during *in vitro* bioassays. The larval mortality rate was measured 12 and 21 d.a.i. of *D. saccharalis* larvae fed with an artificial diet supplemented with 10^8 CFU/mL of *Bacillus* full sporulated cultures amended with penicillin G (100mg/L). The mortality rate was represented as the mean percentage of dead larvae for six replicates. Each replicate contained 10 larvae. Values with the same letter were not significantly ($P > 0.05$) different according to Tukey's test.

2.3.3.3. *Helicoverpa armigera* bioassay

At 7 d.a.i., HD1 (DiPel®) culture induced higher mortality rate into *H. armigera* larvae (95%) than RZ2MS9 culture (30%) and Broth (Control) (13.3%). Nevertheless, at 21 d.a.i. RZ2MS9 and HD1 cultures were statistically similar (90% and 100%, respectively) and significantly higher than Control (18.3%) (Figure 4).

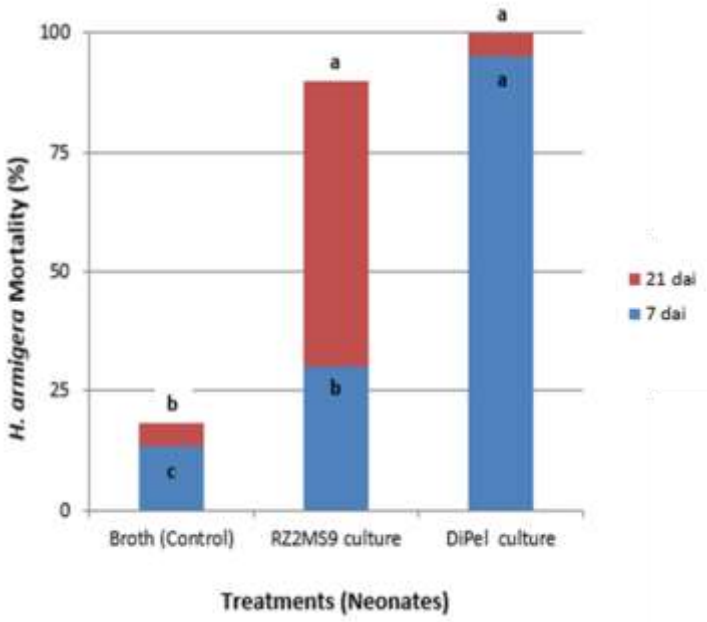

Figure 4. Effects of RZ2MS9 and HD1 sporulated cultures on neonates *Helicoverpa armigera* larvae during *in vitro* bioassays. The larval mortality rate was measured 7 and 21 d.a.i. of *H. armigera* larvae fed with an artificial diet supplemented with 10^8 CFU/mL of *Bacillus* sporulated cultures amended with penicillin G (100mg/L). The mortality rate was represented as the mean percentage of dead larvae for six replicates. Each replicate contained 10 larvae. Values with the same letter were not significantly ($P > 0.05$) different according to Tukey's test.

2.3.3.4. *Spodoptera frugiperda* bioassay

The mortality of *S. frugiperda* newborn larvae fed up with RZ2MS9 culture differed significantly from the Control at 7 and 21 d.a.i. according to Tukey's test, but were statistically lower than DiPel culture mortality rates in the same periods (Figure 5). Despite of that, the analysis of the moths produced in this essay showed a significant occurrence of severe wing deformities in RZ2MS9 treatment (Figure 6).

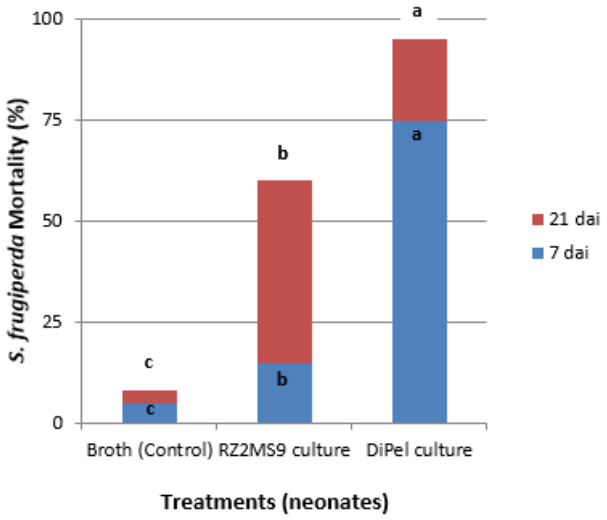

Figure 5. Effects of RZ2MS9 and HD1 sporulated cultures on neonates *Spodoptera frugiperda* larvae during *in vitro* bioassay. The larval mortality rate was measured 7 and 21 d.a.i. after larvae feeding to an artificial diet supplemented with 10^8 CFU/mL of *Bacillus* full sporulated cultures supplemented to penicillin G (100mg/L). The mortality rate was represented as the mean percentage of dead larvae for six replicates. Each replicate contained 10 larvae. Values with the same letter were not significantly ($P > 0.05$) different according to Tukey's test.

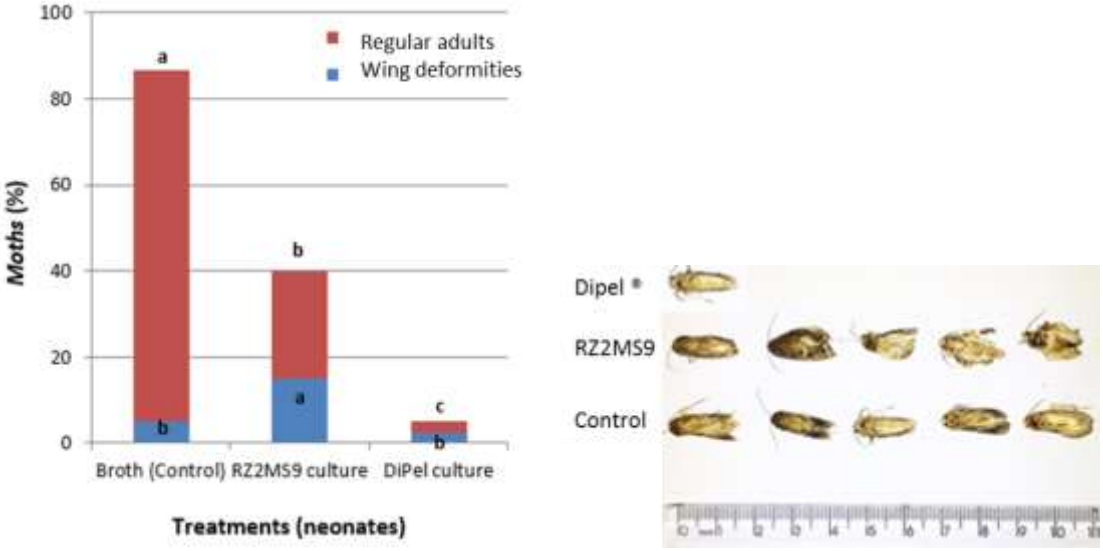

Figure 6. Effects of RZ2MS9 and HD1 sporulated cultures on *S. frugiperda m*oths during *in vitro* bioassay. Left: The percentage of wing deformity was represented as the mean percentage of moths with wing abnormalities for six replicates of ten individuals each. Normal moths were shown in red. Values with the same letter were not significantly ($P > 0.05$) different according to Tukey's test. Right: randomly chosen moths produced by larvae fed up with "DiPel culture", "RZ2MS9" culture and Control were shown, highlighting the wing deformities possibly induced by RZ2MS9 presence in the artificial diet.

2.3.3.5. *Agrotis ipsilon* bioassay

The mortality of *A. ipsilon* newborn larvae fed up with RZ2MS9 culture and HD1 differed significantly from the Control at 60 d.a.i. (Figure 7) according to Tukey's test.

In addition, the analysis of the moths produced in this essay showed a significant occurrence of severe wing deformities in RZ2MS9 and HD1 treatments.

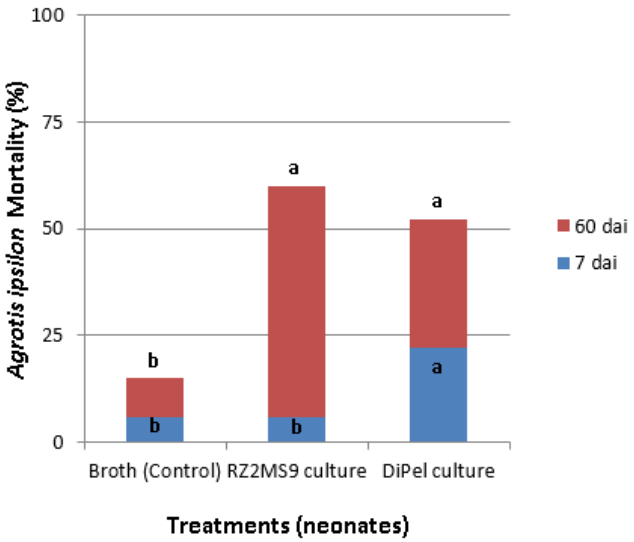

Figure 7. Effects of RZ2MS9 and HD1 sporulated cultures on newborn *Agrotis ipsilon* larvae during *in vitro* bioassays. The larval mortality rate was measured 7 and 21 d.a.i. after newborn larvae feeding to an artificial diet supplemented with 10^8 CFU/mL of *Bacillus* RZ2MS9 and HD1 sporulated cultures amended with penicillin G (100mg/L). The mortality rate was represented as the mean percentage of dead larvae for six replicates. Each replicate contained 10 larvae. Values with the same letter are not significantly ($P > 0.05$) different according to Tukey's test.

2.3.3.6. *Anthonomus grandis* bioassay

The mortality of *A. grandis* newborn larvae fed up with RZ2MS9 culture and HD1 differed significantly from the control at 45 d.a.i. (Figure 8) according to Tukey's test.

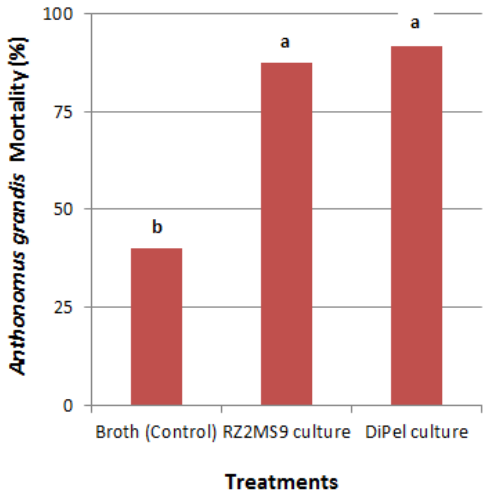

Figure 8. Effects of RZ2MS9 and HD1 sporulated cultures on ecloded *Anthonomus grandis* larvae during *in vitro* bioassays. The larval mortality rate was measured at 45 d.a.i. after newborn larvae feeding to an artificial diet supplemented with 10^8 CFU/mL of *Bacillus* RZ2MS9 and HD1 sporulated cultures supplemented to penicillin G (100mg/L). The mortality rate was represented as the mean percentage of dead larvae of five replicates. Each replicate contained 10 larvae. Values with the same letter are not significantly ($P > 0.05$) different according to Tukey's test.

2.3.4. *In silico* entomopathogenic potential profiling

Genomic mining performed with RZ2MS9 *draft* genome and HD1 genome detected seven major entomopathogenic traits (Table 2). Both RZ2MS9 and HD1 harbored one urease operon with identical proteins, one copy of phospholipase C coding gene, three copies of Immune inhibitor A coding gene, and indentical chitinase coding genes (*chiA1*, *chiD*, *chbG, nagA* and *nagB*). Remarkbly only RZ2MS9 harbored one chitosanase coding gene (*chP*) and an operon which coded for hydrogen cyanide biosynthesis. Only HD1 harbored a gene coding for hemolysin II (Table 2).

Table 2: Genomic profiling displaying *Bacillus* sp. RZ2MS9 and *B. thuringiensis* serovar *kurstaki* HD1 genes and coded proteins with entomopatogenic effect.

Gene/ operon	Name	RZ2MS9	HD1
Urease operon	*ureD*; urease accessory protein	OGY05489.1	AIE34901.1
	ureG; urease accessory protein	OGY05490.1	AIE34902.1
	ureF; urease accessory protein	OGY05491.1	AIE34903.1
	ureE; urease accessory protein	OGY05492.1	AIE34904.1
	ureC; urease subunit alpha	OGY05493.1	AIE34905.1
	ureB; urease subunit beta	OGY05494.1	AIE34906.1
	ureA; urease subunit gamma	OGY05495.1	AIE34907.1
Hydrogen cyanide synthesis	*HcnC*; Hydrogen cyanide synthase subunit	OGY02447.1	-
	HcnB; Hydrogen cyanide synthase subunit	OGY02449.1	-
	HcnA; Hydrogen cyanide synthase subunit	OGY02450.1	-
	HcnB; Hydrogen cyanide synthase subunit	OGY02451.1	-
Phospholipase	*plcC*; phospholipase C	OGY01714.1	AIE31891.1
Hemolysin	*hblAB* hemolysin BL-binding component	OGY05027.1	AIE33900.1
	hlnIII; hemolysin III	OGY01626.1	AIE33539.1
	hlnIII; hemolysin III	OGY03230.1	AIE36776.1
	hlnIII; hemolysin III	-	AIE36778.1
	hlnII, hemolysin II	-	AIE32385.1
Immune inhibitor	*ina;* immune inhibitor A	OGY01720.1	AIE31883.1
	ina; immune inhibitor A	OGY02592.1	AIE32640.1
	ina; immune inhibitor A	OGY04058.1	AIE34026.1
Chitinases	*chiA1*; Chitinase A1	OGY02145.1	AIE31651.1
	chiD; Chitinase D	OGY05605.1	AIE34993.1
	chbG; chitooligosaccharide deacetylase	OGY02773.1	AIE36512.1
	nagA; N-acetylglucosamine-6-phosphate deacetylase	OGY06744.1	AIE35334.1
	nagB; glucosamine-6-phosphate deaminase	OGY05924.1	AIE35333.1
Chitosanases	*chP*; chitosanase	OGY02301.1	-

2.3.5. *In vitro* plate essays

RZ2MS9, HD1 and JL68 showed chitinolytic activity as shown by solubilization index (SI) calculated from halum formed (Figure 9A). RZ2MS9 chitinolytic index was higher than HD1 and JL68 which might be related to combined effect of chitinases and chitosanase (Table 1). RZ2MS9 showed greater proteolytic activity than HD1 (Figure 9B).

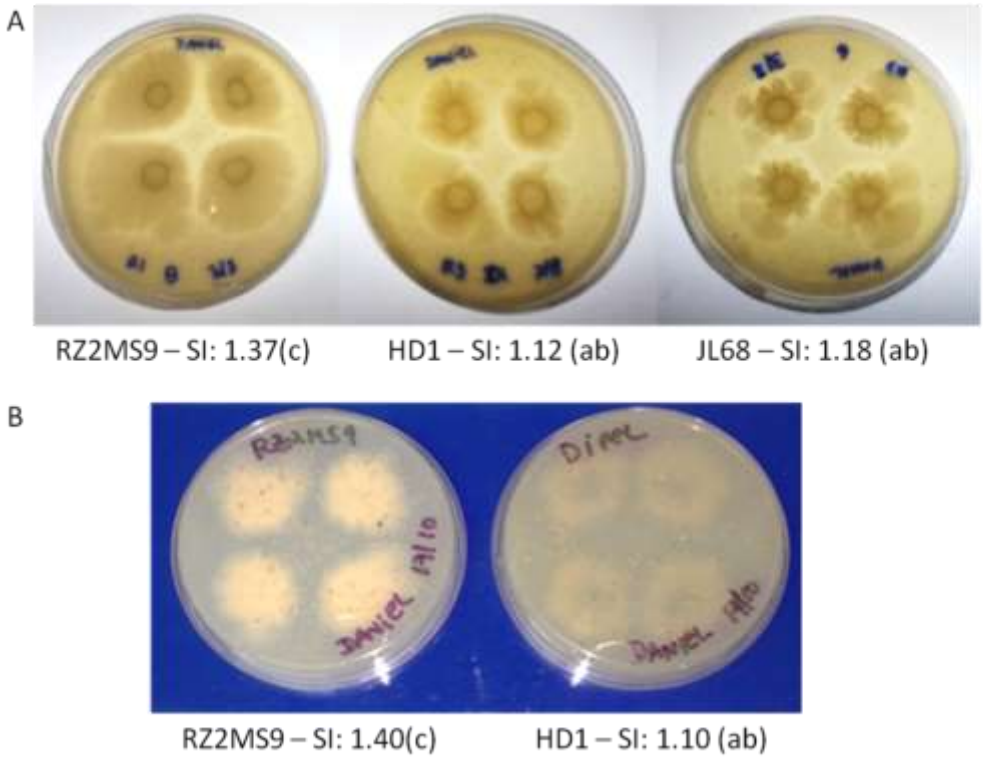

Figure 9. *In vitro* plate essays performed with *Bacillus* spp. A: Chitinolytic activity was evaluated for *Bacillus thuringiensis* strains RZ2MS9, HD1 and JL68, using M9 medium with deacetilated chitin as sole carbon source. B: Proteolytic activity of *Bacillus* strains RZ2MS9, HD1 was accessed using powder milk medium. Solubilization indexes were shown in front of strain name. Different letters indicated different groups according to Duncan's multiple range test.

2.4. DISCUSSION

The use of microorganisms in biological control progressed over the years as an alternative to chemical control due to its broad spectrum of biocidal activity toward the pests, and safeness to human, insect pollinators and the environment (Navon, 1993; Berg et al., 2011; Chowdhury et al., 2015).

Among the exponents of this process, the *B. thuringiensis* strains such as bioinsecticide DiPel® (*B. thuringiensis* serovar *kurstaki* strain HD1) are greatly used in fields (Bravo et al., 2011). The exchanging agricultural market demands for new biological products and high cost of application had favored the search of novel bacterial strains capable of favoring plant growth and yield (Berg et al., 2011; Sansinenea, 2012). In previous work our group investigated the taxonomy of RZ2MS9 using robust phylogenetic constraints such as housekeeping genes and functional genomics. It was observed that RZ2MS9 clustered with *Bacillus thuringiensis* entomopathogenic strains. As *Bacillus* spp. species classification is a complex subject, more data was needed to corroborate the hypothesis of RZ2MS9 being a *B. thuringiensi*s strain brought by phylogenetic results (Batista, 2017).

Remarkably, *B. thuringiensis* strains are primarily used for the arthropods and worms biocidal capacity of its crystalline proteins (Cry proteins), hence the production of this proteins is well studied and is currently used for strain classification purposes (Lerecluz et al., 1993; Bravo et al., 2011; Sansinenea, 2012). Thus, one of major findings was the production by RZ2MS9 at late sporulation phase of two types of Insecticidal Crystalline Proteins (ICPs), one spherical and other cuboid. At the best of our knowledge, the spherical crystals had been related to pathogenicity against insects of Coleoptera and Diptera orders (Höfte & Whiteley 1989; Noguera & Ibarra, 2010; Silva et al., 2010). RZ2MS9 also was able to produce cuboid crystals which were described for both recombinant Cry1I protein and the entire Cry2 proteins group (Höfte & Whiteley 1989; Martins et al., 2008; Silva et al., 2010). A recombinant Cry1I protein was associated to toxicity against cotton boll weevil (*Anthonomus grandis*) (Martins et al., 2008) and Cry2 proteins were entomopathogenic against Coleoptera, Lepidoptera, Diptera larvae (Höfte & Whiteley, 1989).

Bipyramidal crystal found in HD1 sporulated culture is extensively related to Lepidoptera pathogenicity (Höfte & Whiteley 1989) and was used as positive control for ICPs production under the conditions adapted in this work. Besides the evidence of ICP production under microscopy were proved by PCR amplification of RZ2MS9 and HD1 plasmids using degenerated primers, and some of RZ2MS9 sequences did not hit with other available cry sequences in NCBI, suggesting the detection of novel cry coding sequences. Also, RZ2MS9 and HD1 plasmids showed similar patterns according to *B. thuringiensis* megaplasmids (Reyez-Ramirez, 2008).

Despite of importance of *B. thuringiensis* produced ICPs morphological evaluation for taxonomy purposes, the existence of different entomopathogenic effects for similar shaped crystals justified the use of additional strategies to obtain more precise results concerning the production of ICPs by RZ2MS9. Among them, the PCR performed with two different sets of degenerated primers that flank conserved regions in cry protein coding genes was chosen as fast way to aggregate to RZ2MS9 entomological potential. One of such primers sets specifically harbored cry1 protein coding genes (Juaréz-Pérez et al., 1997), entomopathogenic mostly to Lepidoptera and also Coleopera pest larvae (Höfte & Whiteley, 1989; Martins et al., 2008; Quecine et al., 2014). The presence of faint cry coding genes bands using RZ2MS9 and HD1 total DNA as templates might be related to presence of few bacterial plasmid copies in total DNA templates whose extraction procedure favored chromosomic DNA retrieve.

The use of plasmid-enriched DNA templates surpassed this issue and allowed amplification of target bands. Hence, a plasmid was described for the first time for RZ2MS9 in this work.

Interestingly, two sequenced cry coding genes fragments obtained from PCR performed with cry1 degenerated primers using RZ2MS9 and HD1 plasmids retrieved high identities (67% and 85%) comparing to *Bacillus thuringiensis* protein Cry1B (N. acess:KJ619662.1) and *Bacillus thuringiensis* Cry1Ia delta-endotoxin gene (Partial Cds) (N. acess:KJ619662.1) respectively, corroborating optical microscopy ICPs screening results.

B. thuringiensis crystal protein production occured during sporulation phase (Bravo et. al., 2011; Siegwart et. al., 2015). As crystal proteins accumulate in spore mother cells in a high rate before release into extracellular space (Baum et al., 1995), ICP accumulation in culture broth is expected to happen during sporulation justifing whole inoculum (supernatant amended with bacterial cells) in insect feeding assays.

B. thuringiensis isolates are typically used as bioinoculants' active ingredients (Navon et al., 1993; Garczynski & Siegel, 2007). Natural susceptibility of *Bacillus spp.* strains *in vitro* to broadly employed insect diet anticontaminants tetracycline and formaldehyde (Campos, 2017) motivated their replacement in this work by other molecules able to control other microorganisms except the two *Bacillus* strains used. The combined use of penicillin G and ampicillin (100 µg/mL) and streptomycin (50 µg/mL) in insect rearing essays was efficient to avoid diet contamination. The results were also consistent to resistance conferring coding genes found in both of RZ2MS9 and HD1 genomes against penicillin G and ampicillin (OGY02018.1, OGY02421.1, OGY03663.1, OGY05105.1, AIE33778.1, AIE33835.1, AIE34490.1). Nevertheless, streptomycin conferring resistance genes were not recovered from both strains genomes, suggesting their presence in plasmids which complete annotation is currently lacking for both strains.

Sporulated cultures used in larvae-feeding essays remain an important indicator of *B. thuringiensis* entomocidal assessment well correlated to field results (Moar et al., 1990; Sansinenea, 2012). Remarkably, mortality rate levels of *D. saccharalis* larvae fed RZ2MS9 and HD1 strains were statistically similar for both analyzed periods (12 and 21 d.a.i.). As bioinsecticides are usually applied protectively, there would be greater chances to target neonates, and their use could be justified as an attempt to represent field reality (Burges et al., 1983; García-Fraile et al., 2015). As for *S.*

frugiperda essay, RZ2MS9 sporulated culture was able to control significantly more than Broth control, but less than HD1 culture. Despite of that, RZ2MS9 culture treatment was the only to significantly produce most deformed wing moths, which might reduce reproductive fitness of the pest. Such effect could be better understood in future essays.

RZ2MS9 sporulated induced higher mortality rates in *Agrotis ipsilon* larvae and pupae at the end of pest larval cycle than in first instars, suggesting a cumulative pattern of entomocide action by this *Bacillus* strain. Noteworthy, mortality rate levels of *A. grandis* larvae fed with RZ2MS9 and HD1 strains were statistically similar at 45 d.a.i. suggesting a Coleopteran biological control potential described for first time for RZ2MS9 and also HD1. Nevertheless, the insect rearing essays findings corroborated RZ2MS9 cuboid and HD1 bypiramidal crystalline ICP conformations found in this work previously related to Lepidopteran entomopathogenicity. Aditionally, the mortality rate induced by RZ2MS9 treatment against *S. frugiperda*, and *A. grandis* might be connected to cry1B gene activity, considering previous entomopathogenic Cry1B activity against Lepidoptera and coleoptera (Bradley et al., 1995).

As defined by Eilenberg et al. (2001) biological control encloses pest population density suppression and/or impact mediated by a biological agent but also the ability to reduce damage pest can provoke. Moreover, larvae mortality rates observed in RZ2MS9 treatment for sugarcane borer, old-world cotton bollworm and *Agrotis ipsilon* might contribute to reduce next pest generation, and pest overall crop impact.

In such context, secondary entomocidal enzymes contributed to bacterium host colonization and nutrient access and hence delaying of larvae resistance progress against *B. thuringiensis* biochemical machinery (Fedhila et al., 2003; Raddadhi et al., 2007). Among them, HD1 chitinases increased *Spodoptera* and *Plutella* larvae mortality (Wiwat et al., 2000; Arora et al., 2003). Higher chitinolitic activity accessed for RZ2MS9 could be explained by presence of chitosanase in RZ2MS9. Hydrogen cyanide biosynthetic genes presence only in RZ2MS9 constituted the first description outside *Pseudomonas* sp and *Chromobacterium* sp (Short et al., 2018), which might contribute to entomopathogenic potential to be addressed in future studies.

Moreover, the presence of two types of ICPs, secondary biocidal traits such as chitinases, toxins, hydrogen cyanide in RZ2MS9 genome and amplification of uncommon plasmidial ICP coding regions with degenerated primers and diverse original habitat revealed a true entomopathogenic potential richness that surely

reinforces its use a multi-trait plant health promoting strain. Hence, comparing to other *Bacillus thuringiensis* strains we speculate that RZ2MS9 would be a better inoculant choice mainly due to its acknowledged plant growth promotion ability by other mechanisms than biological control, that were further studied in chapter 2 and 3, and other future works.

2.5. CONCLUSIONS

This chapter advanced understanding of benefical relationship between tropical free-living Plant Growth Promoting Rhizobacterium (PGPR) *Bacillus sp.* and plants. Briefly, the detected cuboid and spherical RZ2MS9 Insecticidal Crystal Proteins (ICP) killed *Diatraea saccharalis, Helicoverpa armigera, Agrotis ipsilon and Anthonomus grandis* larvae similarly to commercial bioinsecticide DiPel® (*Bacillus thuringiensis* serovar *kurstaki* HD1) in rearing essays. Additionally, a region with 67% identity to the *Bacillus thuringiensis* Cry1B protein pathogenic against insects of the order Lepidoptera and Coleoptera plasmid was sequenced from RZ2MS9 plasmid. Overall, the first description of RZ2MS9 entomopathogenic potential was provided which corroborated its classification as a *Bacillus thuringiensis* strain and provided clues for future studies in greenhouse and into the field as a novel bioinsecticide.

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3. *IN SILICO* IDENTIFICATION, GENOMIC POSITION AND TRANSCRIPTIONAL PROFILE OF PLANT GROWTH PROMOTING RHIZOBACTERIUM *Bacillus thuringiensis* RZ2MS9 CANDIDATE EFFECTOR GENES INTERACTING TO MAIZE SEEDLINGS

ABSTRACT

Despite of Plant Growth Promoting Rhizobacteria (PGPR) contribution to crop yield, knowledge of intricate symbiotic relationship at molecular level is still scarce. Currently, the study of bacterial effector candidates has increased considerably since they are secreted proteins that can alter plant host development and physiology as well as the plant-microbe interaction. At best of our knowledge, this one of the first initiatives to study bacterial candidate effectors genes involved in mutualistic relationship between a free-living PGPR and its crop host. Previously, free-living PGPR *Bacillus* sp. RZ2MS9 was isolated from *Paullinea cupanea* rhizosphere and showed significant growth promotion ability in maize and soybeans under greenhouse conditions. In this study, we aimed to identify *in silico* candidate effector genes, study their genomic context and to validate their expression during maize interaction. The 12 candidate effector genes identified by the pipeline harbored signal peptide, and lacked transmembrane domain as well lipid surface anchorage. Functional characterization of the candidates using search algorithms NCBI-BLAST, KEGG, Prokka annotation eliminated one RZ2MS9 secreted protein with presumable non-effector function. MEME predicted sixteen motifs in candidate effector proteins sequences. The most relevant categories of predicted subcellular localization according to LocTree3 were cytoplasm (54.5%), apoplast (27.3%), chloroplast (9.1%) and endoplasmic reticulum (9.1%). Six RZ2MS9 candidate effector genes were associated to putative genomic islands suggesting horizontal gene transfer. The candidates OGY04397.1; OGY05372.1; OGY05572.1 were up-expressed exclusively at 12 h.p.i. under gnotobiotic conditions during maize interaction. At 120 h.p.i., two putative cytoplasmic effector proteins were up-expressed (OGY01852.1 and OGY01959.1). OGY05372.1 and OGY05572.1 were up-expressed in all evaluated times. Overall, 81.9% of candidate effector genes transcripts were detected, 45% of them significantly up-expressed in at least one evaluated time corroborating the results from the pipelines and providing new clues for future studies of PGPR and the molecular interaction with crops.

Keywords: Plant-microbe interaction, Candidate effectors, RT-qPCR, Gnotobiotic system, PGPR

3.1. INTRODUCTION

Plant inoculation with beneficial microorganisms, especially Plant Growth Promoting Rhizobacteria (PGPR), has emerged a promising strategy to increase agricultural productivity (Azevedo et al, 2000; Jha et al., 2013; Dey et al., 2014). PGPR can colonize different plant tissues and are native from the rhizosphere, a portion of soil adjacent to the root system rich in organic acids, plant cell debris, root exudates

(Moreira & Siqueira, 2006; Dobelaere et al., 2003). PGPR can stimulate plant health by direct mechanisms such as phytohormone production, nutrient fixation and acquisition, consequently reducing dependence on mineral fertilizers (Ashraf et al., 2011; Bravo et al., 2011). Indirectly, PGPR can add up to crop yield by providing biocontrol against phytopathogens and pests, by active molecules synthesizing and releasing such as siderophores, bacteriocins and insecticidal crystal proteins (Bhattacharyya & Jha, 2012, Dey et al., 2014). The core plant growth promoting mechanisms are shared among main PGPR groups *Rhizobium and Bradyrhizobium, Pseudomonas* and *Bacillus* (Argaw, 2014; Bravo et al., 2011; Dey et al., 2014).

In Gram-positive *Bacillus* spp. the principal plant growth processes include the biological fixation of nitrogen (Ikeda et al., 2013), the solubilization of phosphate (Chen et al., 2006; Idriss et al., 2002; Konietzny & Greiner, 2004; Secco et al., 2017), the production of plant phytohormone auxin (Ali et al., 2009) and ethylene modulation by bacterial ACC deaminase (Bal et al., 2013). *Bacillus* spp also produce siderophores (Szilagyi-Zecchin et al., 2014), insecticidal crystal proteins (Bravo et al., 2007; 2011), other antimicrobial compounds (Choudhary & Johri, 2009; Lanna-Filho et al., 2013) and can induce systemic resistance acquired in plants (Domenech et al., 2006). We also emphasize the production of secondary metabolites, which represent approximately 10% of the sequences of the genome of PGPR *B. amyloliquefaciens* FZB42 (Chen et al., 2007).

Batista et al. (2018) isolated several rhizobacteria associated with guarana plant (*Paullinea cupana*) root. Among them, *Bacillus* sp. RZ2MS9 was able to promote maize growth in relation to the non-bacterized control under greenhouse conditions. In quantitative terms, RZ2MS9 inoculated maize plants collected 30 and 60 days after germination showed increases of 35.5% and 39.4%, respectively, at shoot height, 142.6% and 235.9% increase in aerial part dry weight, and increase of 75.4% and 247.8% of root system dry weight comparing to control.

Despite the high number of commercial inoculants and several crop host developmental and physiological processes ensued by interaction with PGPR, little is known about the molecular mechanisms involved in such interaction (Choudhary & Johri, 2009, Bruto et al., 2014, Choi et al., 2014, Goswami et al., 2016). In this sense, the secretion of proteins, named effectors, is essential for determining the type of interaction between the plant and the microbe. This is because effectors are capable of altering host physiology and development, suppressing plant immune responses

(PTIs) and acting directly on host infection and colonization processes (Abramovitch et al., 2006; Hogenhout et al., 2009; Dalio et al., 2014). Throughout evolution, the plants and microbes developed mechanisms to recognize one another produced molecules and surpass their effect, which establishes the zigzag model (Hogenhout et al., 2009, Jones & Dangl, 2006).

Studies suggest that symbiosis and parasitism would retain "basic compatibility modules" (Kogel et al., 2006) presumably dependent on secretion of bacterial proteins (effectors). Currently, there is a great effort to characterize these effectors, since they provide a better understanding about the specificity with the hosts (Ellis et al., 2009).

The effectors may act in distinct parts of host, for instance apoplastic effectors are exclusively active in the extracellular space, usualy harbor cysteine-rich residues, being more specific (Win et al., 2012; De Jonge et al., 2011). In this sense, some authors include in this group the effectors with enzymatic action, which code hydrolytic enzymes of cell wall degradation (De Jonge et al., 2011). Cytoplasmic effectors act inside the host cell either in the cytoplasm or in organelles (Win et al., 2012). Therefore, many effectors have two main parts: a signal module and a functional portion. The signal module may include different sub-parts: signal peptide, host target signal, and host organelle specific target signal to ensure delivery of the effector at the site of action within the host cell. The secretion of apoplastic effectors rely only on the presence of a signal peptide in the N-terminal portion (Jiang et al., 2011).

Concerning the functional portions of the effectors, they usually have heterogeneous sequences, according to different physiological effects. The C-terminal sequences, in turn, may have different modules. Effector mediated immune responses can occur in a number of ways, from preventing the recognition of PAMPs/MAMPs (Pathogen-Associated Molecular Patterns and Microbial-Associated Molecular Patterns), interruption of signal transduction resulting from recognition, or even direct modulation expression of genes from defense routes (Gust et al., 2007; Gohre & Robatzek, 2008; Felix et al., 2009).

Based on the relevance of effectors concerning to host-microbe interaction, significant plant growth promotion capacity of RZ2MS9 and scarcity of effector studies of free-living rhizobacteria, we performed the *in silico* identification, genomic position and transcriptional profile of Plant Growth Promoting RZ2MS9 candidate effector genes interacting to maize seedlings. First, we used *in silico* prediction tools to obtain RZ2MS9 candidate effectors, then the selected effectors had their genomic

parameters studied, and their expression was quantified by RT-qPCR using maize tissues infected solely with RZ2MS9. Overall, molecular basis of host-beneficial-microbe interaction progressed with our findings as a glimpse of what the research in this system will achieve.

3.2. MATERIAL AND METHODS

3.2.1. Biological material

Bacillus sp. RZ2MS9 was isolated from guaraná (*Paullinea cupana*) rhizosphere at Maues-AM Brazil (Batista et al., 2018). Bacterial colonies were stored in 20% glycerol at -80 °C and routinely cultivated in Luria-Bertani Agar at 28º C. Maize hybrid Pioneer ® P4285H (F1) seeds were kindly provided by prof Dr. Roberto Fritsche-Neto, Allogamous Laboratory, both settled at Genetics Department – University of São Paulo – USP/ESALQ Piracicaba-SP, Brazil.

3.2.2. Effector terms search in RZ2MS9 Draft genome and Prokka Annotation

All 5521 annotated protein coding genes from RZ2MS9 *draft* genome deposited at GenBank database (MJBF00000000) were annotated *de novo* using Prokka rapid prokaryotic annotation tool (Seemann et al., 2014). Both GenBank *draft* genome and Prokka annotated protein coding genes were searched for effector and effector-related terms prior to *in silico* prediction.

3.2.3. Candidate effector genes *in silico* prediction

In silico search for candidate effector genes was performed submitting the FASTA amino acids sequences coded by each annotated gene to a specific pipeline. First, only amino acids sequences that harbored a signal peptide predicted by SignalP V.3.0 software (Bendtsen et al., 2004) were selected. Proteins harboring transmembrane domain (HMM *sprob*>0.5) or anchorage surface were excluded by programs TMHMM V2.0 (Krogh et al., 2001) and GPISom (Fankhauser & Maser, 2005). Only predicted proteins from 4 to 45k Da were considered putative effector proteins.

3.2.4. Candidate effector molecular characterization

The predicted candidate effector genes were submitted to NCBI (National Center of Biotechnology Information) BLASTn (Basic Local Alignment Search Tool) (Altschul

et al., 1990) and KEGG algorithms (Kanehisa et al., 2016) along with retrieved Prokka *de novo* annotation to verify their size and function. The effector candidate proteins were also examined using NCBI Identical Proteins Groups database (https://www.ncbi.nlm.nih.gov/ipg) to search for similar proteins.

Adittionally, selected candidate effector proteins were mapped regarding PFAM domains (e-value <0.01) (Finn et al., 2014) and had their motifs searched *de novo* using deterministic optimization algorithm MEME (Multiple Em for Motif Elicitation) version 5.0.5 (motifs of 6 to 25 amino-acids) (Bailey et al., 2009). The MEME retrieved motifs were submitted to Motif Alignment & Search Tool (MAST) version 5.0.5 from MEME suite to find sequences that matched RZ2MS9 candidate effector proteins motifs. Found motifs that did not were localized in proteins of other organisms than RZ2MS9 were considered exclusive motifs. The subcellular localization of secreted proteins was predicted by the software LocTree3 (Goldberg et al., 2014). All analyses were conducted using default parameters.

3.2.5. Genomic position of candidate effector genes considering putative associated genomic islands

Zisland Explorer software (Wei et al., 2017) was used to predict putative genomic islands considering segmental cumulative Guanine-Citosine (GC) nucleotides profiles from the 33 contigs FASTA sequences of RZ2MS9. For the prediction, the software considered the sequence composition consistence within each island (sequence homogeneity) and sequence relative heterogeneity, that is, the bias GC composition of each island versus the core genome. Recommended higher standard cutoff of 0.25 was employed. The predicted islands and respective flanking regions were manually checked for the presence of mobile genetic elements insertion, such as transposases and integrases, *frameshifts* in the sequence (Zhang & Zhang, 2014; Lu et al., 2016) and hypothetical proteins coding gene content (De Brito et al., 2016) using NCBI genome browser.

3.2.6. Expression analysis

3.2.6.1. Primer Designing

Primer 3 software version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/) was used to design specific sets of primers from nucleotide sequences of the *in silico* selected

candidate effector genes of RZ2MS9 for Reverse Transcription Polymerase Chain Reaction quantitative real time (RT-qPCR). Primer3 and Oligo Analysis Tool software were employed for hairpins and dimers detection during design step (http://www.operon.com/tools/oligo-analysistool). Additionally, primers sequences specificity was tested using NCBI BLASTn tool and Plant Comparative Genomics database Phytozome ® v 12.1 BLAST using deposited maize genomes. Three housekeeping genes with stable and constitutively described expression were used to normalize candidate effector genes expression: *udp*, *fabI*, and *rpoB* (Teixeira, 2017).

3.2.6.2. Target genes

Primers designed in this work for specific detection of candidate effector genes are shown in table 1. Three *Bacillus* housekeeping genes with stable and constitutively described expression were used to normalize candidate effector genes expression: *udp*, *fabI* and *rpoB* (Teixeira, 2017; table 1).

Table 1 – List of specific primers designed for RZ2MS9 candidate effector genes amplification consensus sequences. Length of amplicons is also shown in base pairs and reference. Three housekeeping genes employed for normalization of expression were also displayed (Teixeira, 2017).

Sequence target	Primer	5'→ 3' Sequence	Amplicon Size (bp)	Reference
		***Bacillus* reference genes**		
UDP-N-acetylglucosamine 2-epimerase	*Udp*-F	CATACCCAGAAGAGATGAAT	89	Teixeira, 2017
	Udp-R	CTGTAAGTTCGTTGCTGAT		
Enoyl reductase I	*fabI*-F	CATTAGAAGGACAAGAATCA	114	Teixeira, 2017
	fabI-R	GTGCTAGACCGTGAATAGTA		
DNA-directed RNA polymerase subunit beta	*rpoB*-F	AGACAACACAGATAGCACAG	112	Teixeira, 2017
	rpoB-R	GAAGAAACGAGACACAAGTA		
		RZ2MS9 candidate effector genes		
hypothetical protein BHV55_23155	1852-F	GGTACAGGTGCGGCTTTTG	191	This study
	1852-R	CCGTCTAATGCATCCCCACC		
hypothetical protein BHV55_23730	1959-F	GCAGGTCCAGCAGGAAGATT	196	This study
	1959-R	GGTCCCACCGGTTCTACAG		
hypothetical protein BHV55_19545	2949-F	GCTGGGTGTGCAAAGGATAA	151	This study
	2949-R	ACACTGGTTTCTTTAGCGATGA		
Peptidase	3419-F	GCACAGGAAATGGACAAGCT	187	This study
	3419-R	GCCGCTGGATATTTGCTACG		
hypothetical protein BHV55_12370	4068-F	GCTTTAGGCGGTTTAACGGT	196	This study
	4068-R	AGCGCACATCTTCAGCTCTA		
hypothetical protein BHV55_14100	4397-F	TTGGAGCAACATCAGCAAGT	174	This study
	4397-R	CCTTTCCTGCTGCTAACGAT		
Agmatine deiminase	5340-F	TGAGGGTGAGAAAGTCCACA	195	This study
	5340-R	ATTGGTCCACTGTCCCTTGC		
hypothetical protein BHV55_02500	5372-F	AGCACCTTTGTTTCGTCACA	193	This study
	5372-R	GCCTGTATATAGCCCTTAAAGCC		
DUF5065 domain-containing protein	5572-F	CAACCACAAATTCAGCGTGC	186	This study
	5572-R	ACGTCTGTGTTAAACGTTCCT		
peptigoglycan-binding protein LysM	5640-F	GAACGATACACTTTGGGGCA	158	This study
	5640-R	TGGCGTTTTGAGGAACAGTG		
hypothetical protein BHV55_09325	6682-F	TGGTGTGAAAACAAAAGCAGC	118	This study
	6682-R	TGCGTTAGAAGGAAGTGGAAG		

3.2.6.3. Conventional Polymerase Chain Reaction (cPCR)

Primers specificity was verified by Polymerase Chain Reactions (PCR) made with the following templates: *Bacillus* sp. RZ2MS9 DNA (positive control) and Pioneer P4285H aseptically cultivated maize seedlings DNA as a negative control. Bacterial DNA was extracted using DNeasy ® Blood & Tissue Kit (QIAGEN), DNA of sterile

cultured maize seedlings was extracted using DNeasy ® Mini Plant Kit (QIAGEN) as recommended by the manufacturer.

PCR reactions were optimized in Profex® PCR System thermal cycler (Applied Biosystems) in 25 µL reactions: 1x Taq DNA Buffer, 3.5 mM MgCl2, 0.2 mM DNTP, 5 pmol of each primer (forward and reverse), 1.25 U of Taq DNA polymerase (Thermo Scientific), 50 ng of template DNA and Milli-Q water (qsp). The PCR cycles consisted in initial denaturation step at 95 °C for 2 min, 40 denaturation cycles at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s, and final extension step at 72 °C for 10 min. PCR amplification products were quantified in BioDrop DUO® In-built Sample Port and electrophoresed in 2% agarose gel, purified with Illustra GFX ® PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequenced by Human Genome and Stem Cells Research Center University of São Paulo, São Paulo – SP, Brazil. Specificity was confirmed by comparison of amplicons and the original candidate effector sequences using the MEGA 6.0 (Tamura et al., 2013).

3.2.6.4. RZ2MS9 gnotobiotic colonization of maize seedlings assay

Pioneer ® P4285H maize seeds were surface disinfested at laminar flow chamber in following sterile solutions: 70% ethanol for 1 min, 2% sodium hypochlorite for 2 min, 70% ethanol for 1 min, and twice washed in sterile deionized water. Seeds were germinated and grown under axenic conditions for 6 days in Petri dishes (90 mm) containing triple layered Germitest® paper disks soaked in 3 mL sterile deionized water. Contaminated Petri dishes were eliminated. Six days-old germinated axenic maize seedlings were transfered to 50 mL Falcon® tubes containing 7 mL of Murashige-Skoog (MS) pH 6.0 with half of sucrose content to mimic rhizosphere oligotrophy (Ramos et al., 2000; Trigiano & Gray, 2010). RZ2MS9 cells (final concentration of 10^8 CFU/mL) grown in Luria Bertani broth for 9 h at 28 ºC (180 rpm) were directly added into MS broth. The falcons were kept half closed and wrapped in Parafilm® to allow only gas exchanges with external atmosphere for 7 d under 15 h of photophase. Control-treated and bacterized seedlings were collected 1, 12, and 120 h.p.i. and immediately frozen in liquid nitrogen.

3.2.6.5. Reverse Transcription Polymerase Chain Reaction quantitative real time (RT-qPCR)

Maize seedlings inoculated with RZ2MS9 in gnotobiotic system collected 1 h.p.i., 12 h.p.i. and 120 h.p.i. were immediately frozen in liquid nitrogen and kept in ultrafreezer (-80ºC) until RNA extraction. For the extraction, seedlings were ground into a fine frozen powder using nuclease-free mortar, pestle and liquid nitrogen. Total RNA was extracted with the RNeasy Plant Mini Kit (QIAGEN) using manufacturer's conditions and RNase-free DNase treatment in-column (QIAGEN). Concentration and quality of total RNA were measured in BioDrop DUO® In-built Sample Port and 1% agarose gel electrophoresis. The cDNA synthesis was done using RevertAid® H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) in Profex® PCR System thermal cycler (Applied Biosystems). The reactions were performed according to the manufacturer's conditions: 4 µg of total RNA, 5 pmol of random primer, 4 µL of Reaction Buffer (5X), 1 mM of dNTP mix, 20 U of RiboLock® RNase Inhibitor, 200 U of RevertAid H Minus Reverse Transcriptase and RNase-free water qsp in a final volume of 20 µL.

The qPCR reactions were performed in an Applied Biosystems 7300 Real-Time PCR System in using GoTaq® qPCR System (Promega). Technical duplicates of the four biological replicates were employed for three collection times (1, 12, 120 h.p.i.) (Bustin et al., 2009). Each reaction consisted of 6.25 µL GoTaq® master-mix, 3.625 µL nuclease-free water, 0.125 µL CXR Reference Dye, 10 pmol of each primer and 2 µL of template cDNA (250 ng/µL), into a final volume of 12.5 µL. The qPCR parameters were 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 58 °C for 45 s, and standard equipment dissociation stage for melting curve.

3.2.6.6. Statistical analysis

The efficiency of housekeeping and candidate effector genes expression was calculated by LinRegPCR v.11.0. Relative expression data was calculated by PFAFFL method using Relative Expression Software Tool (REST) (Pfaffl, Horgan & Dempfle, 2002). Differential expression was checked by Pairwise Fixed reallocation Randomization Test (2000 bootstraps).

3.3. RESULTS

3.3.1. *In silico* analysis of effector candidates

Fourteen sequences were predicted as secreted proteins due to simultaneous presence of signal-peptide (predicted by SignalP), absence of both transmembrane domain and surface lipid anchorage (indicated respectively by TMHMM and GPISom programs). After the size mining, 12 sequences were predicted coding a 4 to 45kDa protein. The 12 selected effector candidates were functionally analyzed using from NCBI BLAST, KEGG, and Prokka *de novo* annotation (Table 2). The genic product of eight candidate effector genes were annotated as hypothetical proteins (OGY01852.1, OGY01959.1, OGY02949.1, OGY04068.1, OGY04397.1, OGY05372.1, OGY05572.1, OGY06682.1). Four candidate effectors coded known proteins, 3 with possible effector function (peptidase OGY03419.1, ammonia producing extracellular deiminase OGY05340.1 and peptidoglycan binding cell-wall hydrolase OGY05640.1) and 1 gene coded a beta-lactamase enzyme OGY05297.1, which were not further investigated in expression analysis (Table 2).

Table 2- *Bacillus* sp. RZ2MS9 secreted proteins and their respective coding genes functional study for *in silico* prediction of candidate effector coding genes. Only chosen candidate effector coding genes predicted by first pipeline with aminoacids coded sequences from 4 to 45kDa were displayed. After data mining using NCBI BLAST tool, KEGG and de novo Prokka Annotation, 11 of 12 genes were selected for expression analysis (displayed in bald font).

Protein GenBank access	AA N.	contig nº	Position and orientation	BLAST P	KEGG	Prokka *de novo* annotation
OGY01852.1	**96**	**7**	**6112 to 6399 (-)**	**MULTISPECIES: DUF4183 domain-containing protein [*Bacillus*]**	**-**	**RZ2M29_04610 hypothetical protein**
OGY01959.1	**97**	**7**	**147364 to 147658 (+)**	**hypothetical protein BHV55_23730 *[Bacillus* sp. RZ2MS9]**	**-**	**RZ2M29_04728 hypothetical protein**
OGY02949.1	**175**	**4**	**363117 to 363641 (+)**	**MULTISPECIES: hypothetical protein [*Bacillus*]**	**-**	**RZ2M29_03884 hypothetical protein**
OGY03419.1	**386**	**3**	**366617 to 367774 (-)**	**peptidase [*Bacillus* sp. RZ2MS9]**	**-**	**RZ2M29_03441 hypothetical protein**
OGY04068.1	**154**	**2**	**527812 to 528273 (+)**	**MULTISPECIES: hypothetical protein [*Bacillus*]**	**-**	**RZ2M29_02456 hypothetical protein**
OGY04397.1	**138**	**2**	**860691 to 861104 (-)**	**MULTISPECIES: hypothetical protein [*Bacillus*]**	**-**	**RZ2M29_02795 hypothetical protein**
OGY05297.1	247	1	434463 to 435203 (-)	MULTISPECIES: BcII family subclass B1 metallo-beta-lactamase [*Bacillus*]	*bla2*; metallo-beta-lactamase class B [EC:3.5.2.6]	RZ2M29_00429 Metallo-beta-lactamase type 2
OGY05340.1	**385**	**1**	**476266 to 477420 (-)**	**MULTISPECIES: agmatine deiminase [*Bacillus*]**	***aguA*; agmatine deiminase [EC:3.5.3.12]**	**RZ2M29_00476 Agmatine deiminase**
OGY05372.1	**124**	**1**	**512399 to 512770 (+)**	**MULTISPECIES: hypothetical protein [*Bacillus*]**	**-**	**RZ2M29_00509 hypothetical protein**
OGY05572.1	**154**	**1**	**734187 to 734648 (-)**	**MULTISPECIES: hypothetical protein [*Bacillus*]**	**-**	**RZ2M29_00722 hypothetical protein**
OGY05640.1	**265**	**1**	**815521 to 816315 (+)**	**peptigoglycan-binding protein LysM [*Bacillus* sp. RZ2MS9]**	**N-acetylmura--moyl-L-alanine amidase [EC:3.5.1.28]**	**RZ2M29_00798 Spore cortex-lytic enzyme**
OGY06682.1	**181**	**1**	**1827201 to 1827743 (+)**	**hypothetical protein [*Bacillus* sp. RZ2MS9]**	**-**	**RZ2M29_01863 hypothetical protein**

Considering GenBank Identical Proteins Groups (IPG) database search, 6 of 12 RZ2MS9 predicted effector proteins showed sequence identity higher than 98% to proteins coded by *Bacillus cereus* and *Bacillus thuringiensis* strains (OGY01852.1, OGY04068.1, OGY04397.1, OGY05297.1, OGY05372.1, OGY05572.1); 3 showed

sequence similarity only to *Bacillus cereus* (OGY02949.1, OGY03419.1, and OGY05640.1), 3 were displayed as RZ2MS9 exclusive proteins (OGY01959.1, OGY05340.1 and OGY06682.1).

According to PFam, signal peptides were predicted for all 12 effector candidates, corroborating SignalP results (data not shown). Six PFam domains were found to 5 candidates (e-value<10^{-12})(Table 3).

Table 3- *Bacillus* sp. RZ2MS9 secreted proteins domain type, localization in aminoacids sequence and functional description accessed by Pfam. Only translated candidate effector proteins with Pfam detected domains were displayed. The following RZ2MS9 secreted proteins did not harbor any Pfam domain: OGY01959.1, OGY02949.1, OGY04068.1, OGY04397.1, OGY05372.1, OGY05572.1 and OGY06682.1, and were not displayed at the table.

GenBank access	**Pfam family name**	**Pfam N.**	***E-value***	**Range**	**Short functional description**
OGY01852.1	DUF4183	PF13799	$2.4x10^{-18}$	23-93	DUF: Domain with Unknown Function with conserved ING motif
OGY03419.1	Tranglutaminase-core	PF01841	$5.2x10^{-14}$	139-232	Establishment of intramolecular or intermolecular cross-links in proteins
OGY05297.1	Lactamase B	PF00753	$7x10^{-13}$	69-226	Antibiotics degradation by fungi or bacteria
OGY05340.1	Peptidyl-arginine-deiminase (PAD) *Porphyromonas*	PF04371	$2.7x10^{-90}$	49-381	Deimination of the guanidino group from carboxy-terminal arginine residues of various peptides to produce ammonia and energy.
OGY05640.1	LysM	PF01476	$6.6x10^{-12}$	32-74	The LysM (lysin motif) domain is about 40 residues long with general peptidoglycan binding function; present in some bacterial enzymes involved in bacterial cell wall degradation.
	LysM	PF01476	$3.6x10^{-12}$	99-140	
	Hydrolase 2	PF07486	1.1x10-23	162-263	

MEME *de novo* motifs search identified sixteen motifs ranging from 6 to 21 aminoacids (Figure 1) displayed in 38 sites in 12 effector candidates (Table 4). Start site of each motif found and respective calculated *p-value* were also displayed, providing the probability of finding similarly well-conserved pattern compatible with the motifs in random sequences (Table 4).

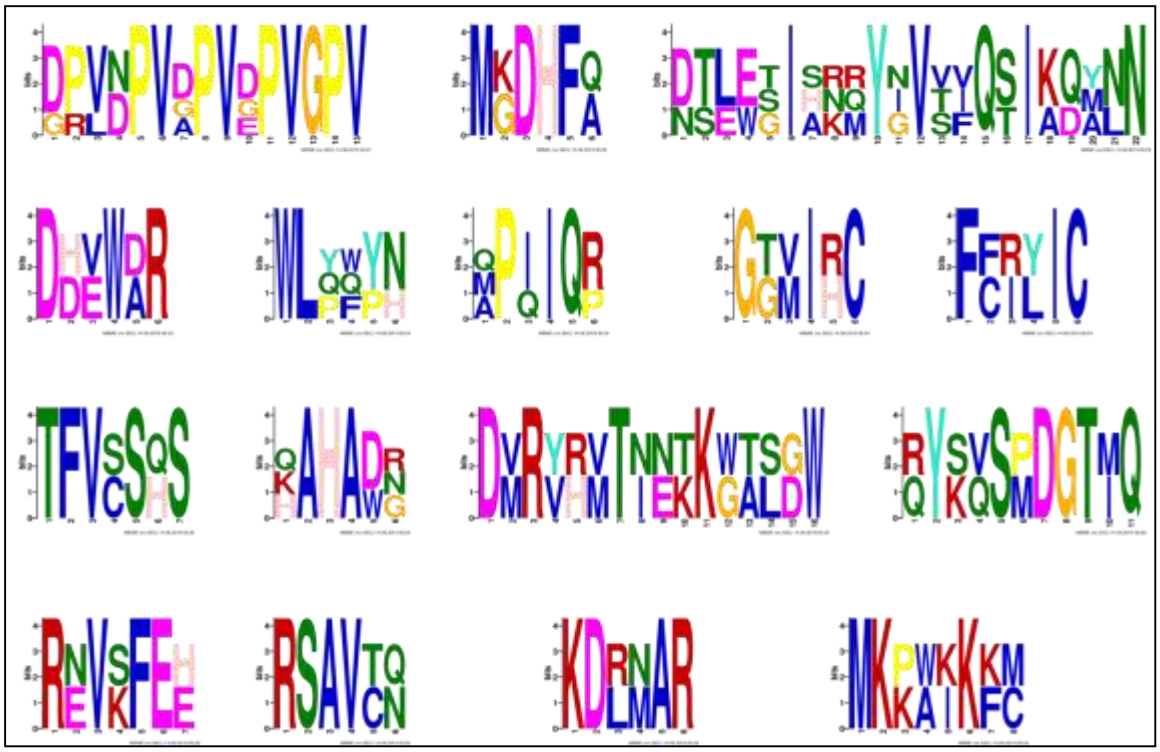

Figure 1- *Bacillus* sp. RZ2MS9 secreted proteins sixteen predicted motifs using deterministic optimization algorithm MEME (Multiple Em for Motif Elicitation) version 5.0.5. Motifs found spanned from 6 to 22 amino-acids.

Same motifs abundance ranged from single to three occurrences across different effector candidates (Table 4). Motif DPVDPVDPVDPVGPV was present only in effector candidate OGY01959.1. Same motif repetition in a single protein was detected for motif DPVDPVDPVDPVGPV found four times in effector candidate OGY01959.1 and for motif DTLESIAKQYGVTIQSIKQYNN found twice in OGY05640.1, and once in OGY02949.1. The most abundant motif found was QPIIQR, harbored in OGY01852.1, OGY04397.1 and OGY05572.1.

Table 4. Motifs location sites and respective flanking regions in RZ2MS9 secreted proteins predicted by deterministic optimization algorithm MEME. RZ2MS9 secreted protein OGY05297.1 had its putative non-effector action highlighted between parentheses. All of other displayed RZ2MS9 proteins were candidate effector proteins. Start site of each motif occurrence, respective calculated *p-value* were also displayed, showing the probability of finding a well-conserved pattern similar to the motif in random sequences.

Name	Start	*p*-value	Upstream	motif location	downstream
OGY01959.1	62	1.14e-19	VDPVDPVGPV	DPVDPVDPVDPVGPV	GPVDPVNPVA
OGY01959.1	47	1.14e-19	VGPVGPVGPV	DPVDPVDPVDPVGPV	DPVDPVDPVD
OGY01959.1	80	3.32e-17	VDPVGPVGPV	DPVNPVAPVEPVGPV	GN
OGY01959.1	29	9.26e-14	EVKVIDAGPA	GRLNPVGPVGPVGPV	GPVDPVDPVD
OGY02949.1	75	3.43e-9	KSKEVDGFLI	MKDPFQ	EASTEQYKNV
OGY05372.1	109	7.24e-9	GYIQASTVWD	MGDPFA	TLYSGTVIRC
OGY05640.1	105	2.00e-25	GQIIYQVQPG	DSLETIARRYNVTVQSIKQMNN	TVGNKLYTGQ
OGY05640.1	38	9.33e-23	ASTIHTVKKN	DTLWGISKQYGVSIQSIKQANN	KGNDQTFIGE
OGY02949.1	153	1.26e-16	QWAIGLSNGG	NTEESIHNMYIVVFQTIADYLN	R
OGY03419.1	261	6.12e-10	FNMSDEQLSK	DHEWDR	SKYPAATTSY
OGY05340.1	129	2.25e-8	EKIDFFIAPT	DDVWAR	DSGPIFVYDS
OGY05340.1	60	1.28e-8	PDEKDKHEGT	WLQWPH	EYTYGEEYKK
OGY05640.1	235	4.44e-8	AISTNGVHSD	WLYFYN	PKTSTDKWIT
OGY05297.1 (Non-effector)	187	5.09e-8	KGHTEDNIVV	WLPQYN	ILVGGCLVKS
OGY05572.1	77	1.76e-8	NKAYEVELRM	QPQIQR	ANGKWENVNK
OGY04397.1	37	1.76e-8	TEGVDNTKNS	APIIQR	TQILKDDFSL
OGY01852.1	1	2.18e-7		MPIIQP	FMASRRFTST
OGY05340.1	370	2.23e-9	GIDVRELYKN	GGMIHC	VTQQQPIALK
OGY05372.1	119	2.62e-8	MGDHFATLYS	GTVIRC	
OGY04068.1	36	8.60e-11	PKAHADGASE	FCRYIC	GPSETLNGFT
OGY02949.1	9	1.49e-8	MKRLSYFL	FFILIC	LVGAGCAKDK
OGY05640.1	18	2.53e-10	HIIPLSAVAI	TFVCSQS	TAEASTIHTV
OGY05372.1	23	4.38e-9	ATLIAGSSLS	TFVSSHS	VQADTLDPNY
OGY05297.1 (Non-effector)	116	1.59e-9	QKRVTDVIIT	HAVADR	IGGIKTLKER
OGY04068.1	27	1.04e-7	GLTVMNIETP	KAVADG	ASEFCRYICG
OGY03419.1	215	1.12e-7	NHIVTGTGNG	QAVAWN	LVNIENKWYH
OGY04068.1	76	4.12e-19	IAKITNDRAE	DVRYRVTIEKKWASGW	GYYETDFNFG
OGY04397.1	68	1.28e-17	EVSFEHVKKA	DMRVHMTNNTKGTLDW	SLKDSKGNVI
OGY03419.1	336	1.10e-15	FAAKPEKVEV	RYKQSMDGTMQ	DIKKVLNEIN
OGY06682.1	78	6.09e-13	LNELSINGNI	QYSVSPDGTIQ	YGVKTKAAKE
OGY04397.1	57	4.96e-10	KDDFSLINGE	REVSFEH	VKKADMRVHM
OGY02949.1	50	4.14e-9	VVGEKPKDTF	RNVKFEE	MKLEELEKKS
OGY03419.1	183	1.74e-9	AYTAYEALAN	RSAVCQ	GYTLLTYELL
OGY05340.1	207	2.87e-7	LDSNGTALLT	RSAVTN	KNRNPDLSEK
OGY03419.1	71	1.36e-8	EENITITYKT	KDRNAR	NIMDQLYGEF

OGY05640.1	149	2.62e-8	KINSSISQKE	KDLMAR	LVTAEAGGES
OGY05572.1	1	2.49e-10		MKPWKKKM	GMTMLASAIA
OGY05340.1	1	4.85e-10		MKKAIKFC	LIATLSTTII

Seven effector candidates were predicted as cytoplasmic (OGY01852.1, OGY01959.1, OGY02949.1, OGY04068.1, OGY05340.1, OGY05372.1, OGY05640.1); two apoplastic (OGY04397.1, OGY05572.1), one putatively directed to chloroplast (OGY06682.1) and other to endoplasmic reticulum (OGY03419.1) (Table 5).

Table 5- *Bacillus* sp. RZ2MS9 secreted proteins subcellular prediction performed with LocTree3 distribution according to putative effector function.

Loc Tree3 Subcelular localization - plant networks	**RZ2MS9 predicted secreted proteins**	
	Candidate Effector function	**Non-Effector function**
Cytoplasmatic	OGY01852.1; OGY01959.1; OGY02949.1; OGY04068.1; OGY05340.1; OGY05640.1	-
Apoplastic	OGY04397.1; OGY05372.1; OGY05572.1	OGY05297.1
Chloroplastidial	OGY06682.1	-
Endoplasmic Reticulum	OGY03419.1	-

3.3.2. Genomic position of candidate effector genes considering putative associated genomic islands

Genomic location of six detected GI was defined according to GC profile (spotted in red, figure 4). G2 average GC content was 4% lower than surrounding genomic borders from RZ2MS9 contig 1 (MJBF01000001.1). On the other hand, GI3 and GI4 average GC content were approximately 4% higher comparing with the rest of RZ2MS9 contig 2 (MJBF01000012.1) and RZ2MS9 contig 4 (MJBF01000028.1). Additionally, GI5 and G6 contiguous GI average GC content was 4% higher than the rest of RZ2MS9 contig 7 (MJBF01000031.1).

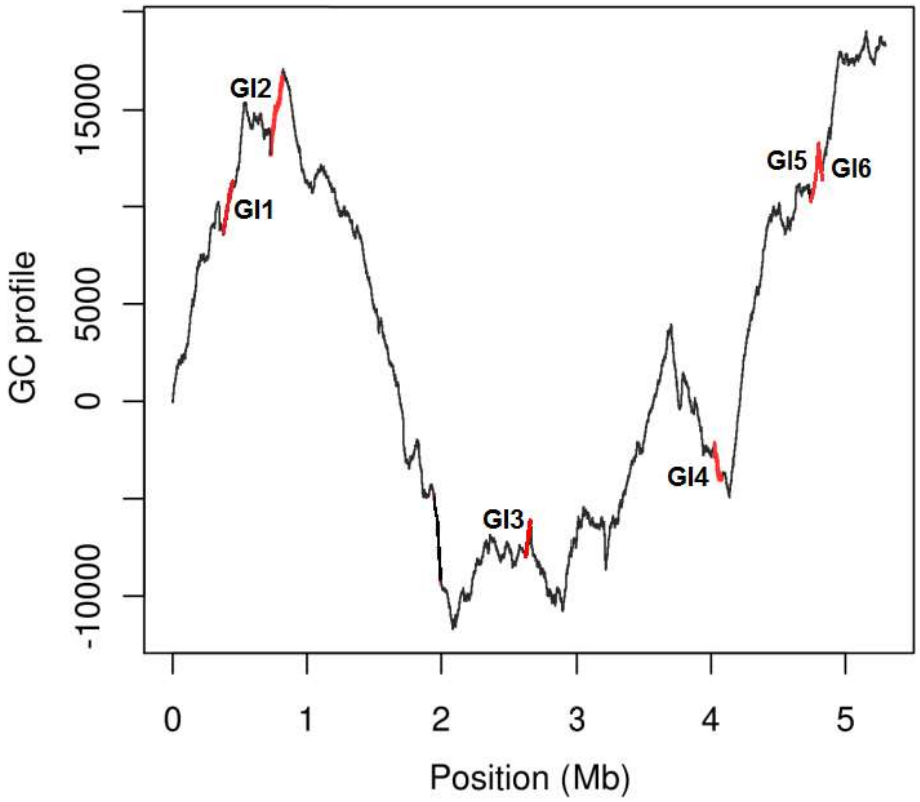

Figure 4 – GC profile skew of RZ2MS9 draft genome sequence. Six putative Genomic Islands (GI) were physically defined by sharp GC content shifts spotted in red (GI1, G2, G3, G4, G5, GI6). Only GI delimited by GC content shifts detected inside the contigs were considered.

All of predicted genomic islands harbored at least one candidate effector gene (Table 6).

Table 6 – General genomic constraints of six predicted Genomic Islands (GI)s in RZ2MS9 draft genome.

Name	Contig Position	Contig	Length (bp)	Coded Genes	Harbored candidate effector proteins	Hypothetical proteins	Plant Growth promoting coded proteins	
							NCBI code	Mechanism
GI1	429755 to 515304	1	85549	81	OGY5340.1	28	OGY05307.1	quorum quenching
							OGY05336.1	polyamine synthesis
							OGY05338.1	polyamine synthesis
							OGY05340.1	polyamine synthesis
							OGY05371.1	Chemotaxis
GI2	732548 to 823186	1	90639	79	OGY05572.1; OGY05640.1	32	OGY05594.1	synthesis of phytormone cytokinin
							OGY05596.1	entomopathogenicity
							OGY05605.1	entomopathogenicity
							OGY05614.1	siderophore
							OGY05615.1	siderophore
							OGY05616.1	siderophore
							OGY05617.1	siderophore
							OGY05618.1	siderophore
							OGY05638.1	Organic phosphorous solubilization
							OGY05639.1	entomopathogenicity
GI3	824914 to 885220	2	60306	68	OGY04397.1	22	OGY04333.1	chemotaxis
							OGY04334.1	chemotaxis
							OGY04365.1	quorum sensing
							OGY04380.1	sporulation
GI4	338485 to 398693	4	60209	67	OGY02949.1	20	OGY02933.1	chemotaxis
							OGY02945.1	sporulation
							OGY02946.1	phosphate solubilization
							OGY02966.1	sporulation
							OGY02982.1	siderophore
							OGY02990.1	sporulation
GI5	55 to 99814	7	99760	79	OGY01852.1	26	OGY01853.1	sporulation
							OGY01885.1	synthesis of phytormone auxin
							OGY01888.1	sporulation
							OGY01895.1	quorum sensing
GI6	100196 to 148297	7	48102	44	OGY01959.1	8	OGY01926.1	sporulation
							OGY01929.1	sporulation
							OGY01930.1	sporulation
							OGY01935.1	nitrogen biological fixation

3.3.3. Expression analysis

All 11 effector candidates selected by *in silico* prediction step were used to RT-qPCR expression analysis.

3.3.3.1. Selected candidate effectors genes and specific primer design

Amplicons sizes of candidate effector set of primers ranged from 118 to 196 base pairs without hairpins and dimers formation. Pioneer® P4285H maize seedlings DNA used as template led to absence of amplification (Figure 5).

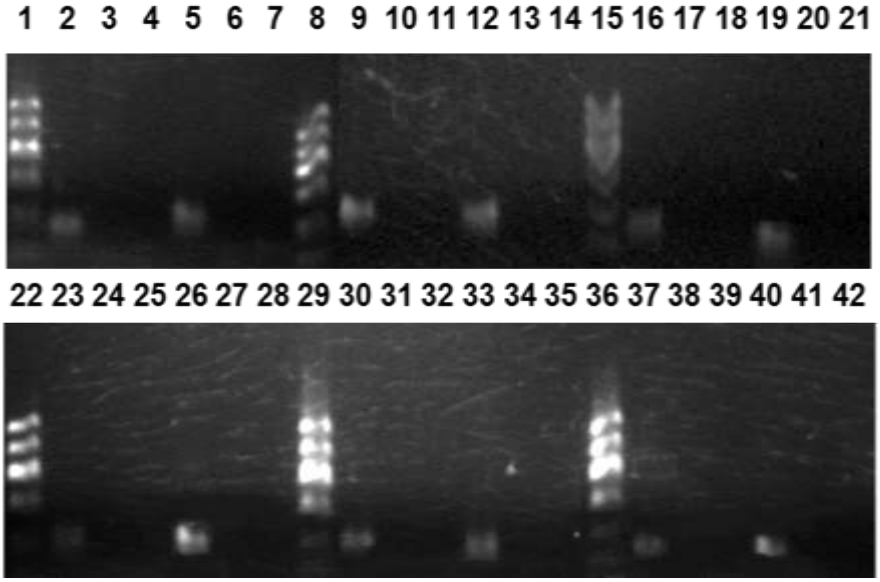

Figure 5 – Electrophoresed PCR results using specific primers designed toward RZ2MS9 candidate effector genes amplification with different DNA templates: 50ng of RZ2MS9 50ng DNA (lanes: 2, 5, 9, 12, 16, 19, 23, 26, 30, 33, 37 and 40); 50ng of Pioneer® P4285H seedlings DNA (lanes: 3, 6, 10, 13, 17, 20, 24, 27, 31, 34, 38 and 41); MilliQ water negative control (lanes: 4, 7, 11, 14, 18, 21, 25, 28, 32, 35, 39 and 42). Molecular ruler 100pb Fermentas®: lanes 1, 8, 15, 22, 29 and 36.

3.3.3.2. Reverse Transcription Polymerase Chain Reaction quantitative real time (RT-qPCR)

Eight RZ2MS9 effector candidates had their expression detected by RT-qPCR (Figure 6). Only the effector candidates coding for the proteins OGY02949.1 and OGY06682.1 were not detected in the expression analysis (Figure 6).

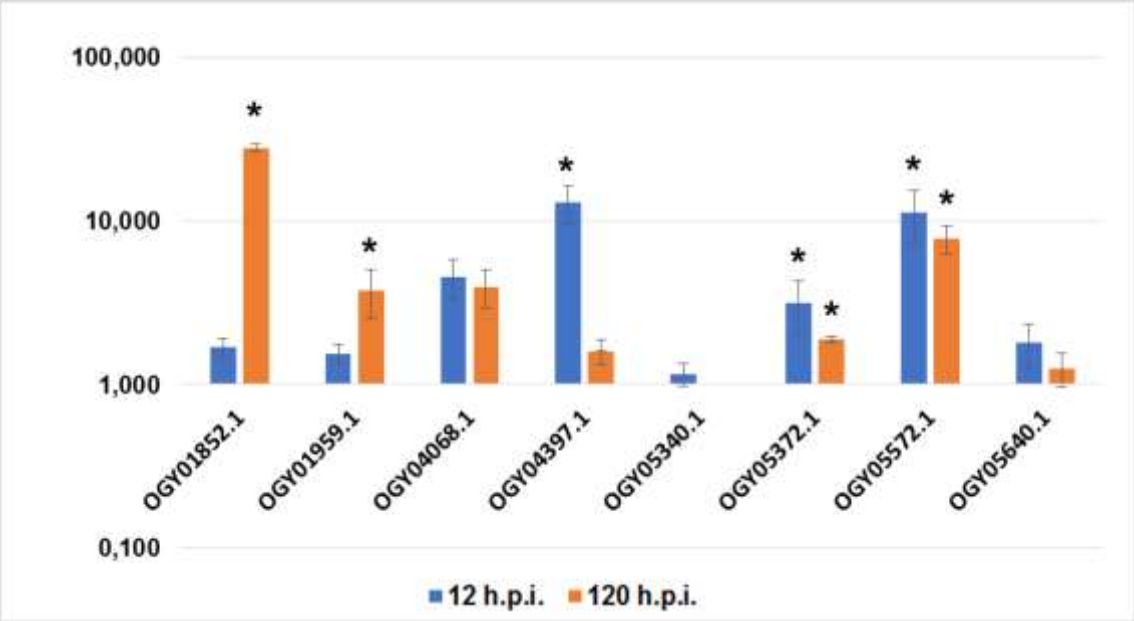

Figure 6 - Candidate bacterial effector genes relative expression data calculated by PFAFFL method of maize P4285H seedlings at 12-1 h.p.i. and 120-1 h.p.i. The gene transcripts were named according to the protein coded by their sequence. Candidate effector genes with significant differential expression ($p<0.05$) comparing to normalizing genes checked by pairwise fixed reallocation randomization test (2000 bootstraps) were spotted with an asterisk. Standard error was calculated considering three biological and two technical replicates.

Effector candidates coding to OGY04397.1, OGY05372.1 and OGY05572.1 were significantly up-expressed ($p<0.05$) at 12 h.p.i, all of them were predicted to be apoplastic effectors. At 120 h.p.i., s OGY01852.1, OGY1959.1, OGY05372.1 and OGY05572.1 were significantly up-expressed ($p<0.05$), showing the contribution of two putative cytoplasmic effectors to maize-RZ2MS9 interaction. Effector candidates coding to OGY5372.1 was down-expressed comparing 12 and 120 h.p.i. (Figure 6).

3.4. DISCUSSION

It is known that during the plant-microbe interaction there is a battle at the molecular level; while hosts try to recognize and prevent entrance and colonization of certain microbes, others putatively suppress the host recognition. The microbe presence is often tolerated by the host due to a co-evolution process (Jones & Dangl, 2006; Grant et al., 2006; Block et al., 2009; Ellis et al., 2009; Newton et al., 2010) In this context, effector molecules must be highlighted, since they can interfere with host physiological processes such as plant defense system (Grant et al., 2006; Block et al., 2009). Effectors allow the host colonization and may contribute to plant health and yield (Jha et al., 2013; Choi et al., 2014).

For instance, phytopathogenic bacteria rely on several secreted proteins delivered by type III secretory system (T3SS) to suppress the plant immune system (Galán & Wolf-Watz, 2006; Grant et al., 2006; Fu et al., 2007; Block et al., 2009; Green & Mecsas, 2016) and the symbiosis between leguminous plants and nitrogen fixing bacteria of the genus *Rhizobium* sp depends on Nodulation Outer Proteins Effectors (Nops) (Soto et al., 2009). In addition, the NopL and NopT candidate effector proteins (from the YopT-AvrPphB family of effectors) are possibly associated with signal transduction in the establishment of the symbiotic relationship, as they are phosphorylated by plant kinase proteins (Bartsev et al., 2004; Skorpil et al., 2005). Moreover, the transient expression of NopT in *Arabidopsis thaliana* triggered chlorosis and necrosis, and in tobacco led to the hypersensitive response (Dai et al., 2008). The candidate NopD effector of *Sinorhizobium fredii* was shown to be similar to *Xanthomonas* spp effector XopD crucial for pathogenicity by interference in the expression of plant defense genes (Hotson et al., 2003). Although its importance in plant–microbe interaction (An et al., 2018) little is known concening the role of effectors from PGPRs, and most of works addressed the legume-Rhizobiales systems (Dai et al., 2008; Soto et al., 2009), which made our work significantly innovative.

It is known that candidate bacterial effectors usually are secreted small proteins without obvious homology to other proteins (Gohre & Robatzek, 2008; Ellis et al., 2009). Additionally, peptide signals should be present, ie peptide sequences conserved in the N-terminal portion typical of the secretory system employed by the pathogen (Schechter et al., 2006). In addition, it is important to note that there is no single criterion to ensure that a candidate actually acts as an effector (Ellis et al., 2009), and use of effector one-step predictors performance is highly limited by available tools for the prediction of bacterial secretion systems, reduced specific amount of information related to effectors available at curated datasets (An et al., 2018).

The performed pipeline identified twelve RZ2MS9 effector candidates harboring signal peptide, lacking transmembrane domains and without GPI anchoring; ten with unknown function in RZ2MS9 *draft* genome (Batista et al., 2016). RZ2MS9 effector candidates had average residues size of 177, which is significantly lower than *Rhizobium (Sinorhizobium)* and *Bradyrhizobium* NOPs symbiotic effectors respective average size of 429 and 407 residues (Uniprot, 2019). Direct search of effector and effector-related terms into RZ2MS9 genome and into Prokka de novo annotation did not retrieve any effector candidate, which can be explained by low sequence

conservation among effectors possibly related to high selection pressure. As a result, little effector similarity is shared with database deposited proteins (Ellis et al., 2009).

A total of five PFAM domains were found in 11 candidates RZ2MS9 selected putative effector candidates: PF13799 in OGY01852.1; PF01841 (tranglutaminase-core) in OGY03419.1; PF04371 (peptidyl-arginine-deiminase) in OGY05340.1; PF01476 (LysM) and PF07486 (Hydrolase 2) in OGY05640.1. PF13799 is a domain with unknown function (DUF4183). Three RZ2MS9 effector candidates have shown enzymatic activity: peptidase OGY03419.1; agmatine deiminase OGY05340.1 and peptigoglycan-binding protein LysM OGY05640.1. PFam domain PF01841 was localized from residues 139-232 of RZ2MS9 protein OGY03419.1. Interestingly, motif RSAVCN located inside PF01841 domain (transglutaminase core) from residues 183 to 188 harbored a cysteine at residue 187, suggesting OGY03419.1 might be a cysteine-type peptidase (Potempa et al., 2005).

OGY05340.1 was selected as putative effector due to its ammonia releasing activity related to deamination of arginine residues which could interfere in host pH homeostasis and physiology (Soares & Knuckley, 2016). RZ2MS9 secreted protein OGY5640.1 was also chosen for expression analysis because double occurrence of peptidoglican binding RZ2MS9 LysM motif (PF01476) was related to similar LysM domain harbored by Ecp6 effector protein of fungal phytopathogen *Cladosporium fulvum* which is responsible for structural carbohydrate chitin scavenging as a strategy to escape host recognition (Bolton et al., 2008; Sánchez-Vallet et al., 2013).

Interestingly motif DPVDPVDPVDPVGPV was found repeated in four sites physically close in candidate effector OGY01959.1 and is physically constituted by four tandem arrays of DPV amino-acids followed by a single GPV repeat,. Such defined structural array have been described for numerous effector proteins and are presumably involved in adaptive evolution or effector-triggered immunity evasion (Bartsev et al., 2004; Skorpil et al., 2005; Grant et al., 2006; Dai et al., 2008; Block et al., 2009; Kim et al., 2009; Dean et al., 2011; Taylor et al., 2012; Mesarich et al., 2015). Motif QPIIQR was found in three RZ2MS9 effector candidate OGY01852.1, OGY04397.1 and OGY05572.1, and motif DMRVRMTNEKKWALGW; none of them previously documented in other works.

Nevertheless, the high microbial population density of the rhizosphere favors the coexistence of bacteria with similar niches, providing a greater frequency of horizontal gene transfer and recombination events, justifying the similarity of host colonization

mechanisms (Bjorklof et al., 2000; Lindow & Brandl, 2003). However, the richness of species and substrates itself makes it difficult to study the role of each individual in complex biological systems such as plant-microbe interaction. One approach to reduce system complexity was the development of gnotobiotic systems, which consists in expose sterile cultivated plant or animal hosts to a microbially controlled environment. That means the host would be exposed only to those microbes that the scientists want to be present in it (Simons et al., 1997; Gamalero et al., 2003; Pommerville, 2017). Thus, gnotobiotic systems have been successfully used for plant-microbe interaction studies (Simons et al., 1997; Gamalero et al., 2003; Müller et al., 2016). Host-microbe dynamics was initially studied in the proposed system, by comparison of seedlings growth and development under the proposed gnotobiotic conditions with and without RZ2MS9 inoculation (Chapter 3).

A total of five GI were predicted and harbored at least one RZ2MS9 candidate effector gene and several hypothetical proteins, and also bacterial genes which code for proteins involved in plant growth promotion. Most of GIs were located next to transposition events, and noteworthy five of six RZ2MS9 effector candidates associated to GIs coded for hypothetical proteins, which corroborate relation between low sequence conservation of several effectors with the respective regions in the genome they are located (Jiang et al., 2011).

The RZ2MS9 effector candidate genes significantly up regulated ($p<0.05$) at 12 h.p.i. (OGY04397.1, OGY05372.1 and OGY05572.1) were predicted to be apoplastic effectors by LocTree3 (Table 5). These findings were consistent since the apoplast constitutes the first layer of host root cells any rhizobacteria must interact to when leaving rhizosphere and entering into the roots. Hence, it is much probable that within a short period of time, 12 h.p.i., these apoplastic bacterial effector proteins have been released in host intercellular spaces to contribute to infection, by inhibition of host recognition or degrading host defense proteins (Ramos et al., 2000; Gohre & Robatezek, 2008; De Wit, 2016).

At 120 h.p.i. RZ2MS9 candidate coding to cytoplasmic effector proteins OGY01852.1, OGY1959.1 (Table 5) and aforementioned putative apoplastic effectors proteins OGY05372.1 and OGY05572.1 were significantly up-expressed ($p<0.05$). By 120 h.p.i. RZ2MS9 cells probably had being in maize tissues, thus it is expected that cytoplasmic effectors contribution have increased during early host colonization by

RZ2MS9 to feed up other processes than infection which improve microbe fitness inside the tissues and access to host metabolic products (Gohre & Robatezek, 2008).

Although the work was carried out in gnotobiotic conditions, it is the first study of effector candidates of a free-living PGPR. The proposed pipeline showed to be efficient to predict candidate effector genes, nine of eleven predicted had their expression detected (81.8%) and five of them were significantly up-expressed. Consequently, the candidate effectors transcriptional profile corroborated the results obtained by *in silico* prediction tools along with genomic parameters evaluation and subcellular localization that provided genetic information for further more detailed studies in PGPR-plant interaction.

3.5. CONCLUSIONS

The first *in silico* identification and transcriptional profile of twelve candidate effector proteins coding genes from a free-living PGPR, *Bacillus thuringiensis* RZ2MS9 during interaction with maize host in a gnotobiotic system was provided. Overall, 81.9% of candidate effector genes transcripts were detected, 45% of them were up-expressed at 12 and/or 120 h.p.i., OGY05372.1 and OGY05572.1 at both times, corroborating the adopted pipeline for *in silico* prediction. Six RZ2MS9 candidate effector genes were associated to putative genomic islands suggesting horizontal gene transfer. Moreover, this chapter contributed to understanding of benefical relationship between Plant Growth Promoting Rhizobacterium (PGPR) *Bacillus sp.* and maize in a molecular way. The predicted effectors provided new clues for future studies of RZ2MS9 molecular interaction with plants and might contribute to understanding of plant growth mechanisms activated by the bacterium.

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4. GENE EXPRESSION ANALYSIS AND GROWTH PROMOTION PARAMETERS OF MAIZE SEEDLINGS (P4285H CULTIVAR) DURING INTERACTION WITH PLANT GROWTH PROMOTING RHIZOBACTERIUM *Bacillus thuringiensis* RZ2MS9

ABSTRACT

Plant Growth Promoting Rhizobacteria (PGPR) inoculation has been emerging as an eco-friendly alternative to keep agricultural yield through host physiological processes enhancement and by microbial biocontrol of pests and phytopathogens. However, the knowledge about transcriptional changes and mechanism related to host-microbial interaction that results in plant growth promotion is scarce. In this chapter, maize-RZ2MS9 benefic relationship was studied considering growth promotion parameters such as chlorophyll, dry matter, soluble sugars content and expression of host genes related to defense, hormonal responses and developmental processes toward understanding of RZ2MS9 contribution to observed phenotype at early interaction *in vitro* and under greenhouse condition. Higher chlorophyll a and b contents and increased fresh and dry matter were observed respectively in leaves and roots of bacterized maize seedlings cultivated in greenhouse harvested in V2 stage. Remarkably, *in vitro* maize leaves and roots gene expression profile reproduced greenhouse results for most of analyzed genes, validating proposed *in vitro* system for further studies. Up-expression of *lox, pr1*, and beta-glucosidase *bglu*60.1 in leaves of bacterized plants suggested activation of host defense mechanisms due to recognition of RZ2MS9. RZ2MS9 modulated the expression of different genes in leaves and roots, comparing to control, favoring roots higher sink strength and growth at early stage V2 expressed by fresh matter, dry matter and soluble sugars increase and higher expression of auxin-responsive gene *iaa14,* sucrose synthase *susy* than control. Down-expression of ciszeatin-transglicosidase in bacterized roots suggested specific release of major maize cytokinin zeatin in the roots. Noteworthy, transcripts of cytokinin microbial biosynthetic gene *miaA* from RZ2MS9 were detected similar levels in leaves and roots of bacterized maize seedlings cultivated in greenhouse and under cytokinin favoring culture conditions *in vitro*. RZ2MS9 contribution to increased chlorophyll content in leaves of greenhouse cultivated maize seedlings might be related to host hormonal balance interference through a mechanism to be further studied in which release of hormone bound forms in roots and leaves by the host, and direct production of cytokinin by the bacterium might participate. Moreover, maize gene differentially expressed during RZ2MS9 interaction might contribute to unserstanding of RZ2MS9 in maize growth promotion and as key gene marker for other maize-PGPR systems studies.

Keywords: Host-microbe interaction, Chlorophyll levels, Invertases, Sink strenght, *miaA*

4.1. INTRODUCTION

Plant Growth Promoting Rhizobacteria (PGPR) can colonize diverse plant tissues and are rhizosphere borne (Moreira & Siqueira, 2006; Dobelaere et al., 2003). PGPR can increase plant health directly by production of phytohormones, fixation of nitrogen,

and acquisition of nutrients, consequently decreasing need for mineral fertilizers (Arkhipova et al., 2005; Ashraf et al., 2011; Koenig et al., 2002; Bravo et al., 2011; Quecine et al., 2012; Jez et al., 2016). Indirectly, PGPR can benefit final culture yield by acting on biocontrol of pests and phytopathogens, by synthesizing and releasing of active molecules such as insecticidal crystal proteins, siderophores, bacteriocins (Dey et al., 2014; Großkinsky et al., 2016). The main PGPR groups include *Pseudomonas, Rhizobium and Bradyrhizobium, Azospirillum* and *Bacillus* (Preston, 2004; Glick et al., 2012; Dey et al., 2014; Goswami et al., 2016).

Batista et al. (2018) isolated several rhizobacteria associated with Amazon rainforest guarana plant (*Paullinea cupana*) root system. Among them, *Bacillus thuringiensis* RZ2MS9 was proved to be an endophytic (Almeida, 2018), has had its genome sequenced (Batista et al., 2016) and was able to promote maize growth comparing to control treatment under greenhouse conditions (Batista et al., 2018).

While there have been significant advances in elucidating the details of plant-bacterial interactions (Bhattacharyya & Jha, 2012; Bruto et al., 2014, Choudhary & Johri, 2009; Hungria et al., 2010; Choi et al., 2014, Goswami et al., 2016), essential points about these processes remain to be further studied. For instance, to what level is primary plant host metabolism changed by PGPR and which transcriptional alterations take place during the beneficial interaction (Schwachtje et al. 2011, Choi et al., 2014).

Endophytic bacteria induced plant immune signaling and host hormonal responses (Verhage, Wees & Pieterse, 2010; Plucani do Amaral et al., 2014). During biotic interaction, expression of plant *lox1* and *pr1* were connected respectively to jasmonic acid and saliciclic acid defense responses (Gond et al., 2015; Ding et al., 2017). RBOH (respiratory burst oxidases) NADPH oxidase protein homologues were related to reactive oxygen species production (Zhang et al. 2006; 2010).

The modulation of auxin and cytokinin levels is associated to early stages of plant growth and development (Taiz et al., 2017). Both hormones may act in production sites and in other plant organs. Major plant growth and development changes rely on the combined effect of cell division stimulated by cytokinin and elongation stimulated by auxin, the amount of each hormone present in the tissue in active form at a specific time (Taiz et al., 2017). However few studies evaluated host response to microbial inoculation at transcriptional level (Plucani do Amaral et al., 2014; Gond et al., 2015; Ding et al., 2017; Espindula et al., 2017).

In bacteria, tRNA degradation by isopentenyl transferase MiaA is considered a marker of microbial cytokinin production through the release of *cis*-zeatin riboside and/or methylthiolzeatin (Cherayil & Lipset, 1977; Morris et al., 1981; McGaw & Burch, 1995; Koenig et al 2002; Patel et al., 2012; Großkinsky et al., 2016). Also, *Shigella flexneri* mutants lacking *MiaA* showed virulence-related regulator VirF inefficient translation (Durand et al., 2000) suggesting a role for *miaA* in host-bacterial pathogen relationship. At best of our knowledge few studies evaluated microbial *miaA* at transcriptional level during host interaction. *MiaA* expression associated to cytokinin synthesis was described in free-living nitrogen fixing PGPR *Pseudomonas stutzeri* A15 colonizating rice (Rediers et al. 2003), and *Pseudomonas fluorescens* G20-18 in *Arabidopsis* (Großkinsky et al., 2016). Moreover, *miaA* is present in RZ2MS9 genome (MJBF00000000; locus tag BHV55_03700).

In this chapter we evaluated the RZ2MS9 ability to modulate the expression of *Zea mays* cv. P4285H (V2 stage) genes involved in biotic interaction and response to colonizing bacteria, growth, sucrose metabolism, and photosynthesis. Most of them are regulated by plant hormones levels and represented canonical physiological proceses that favor final yield, such as root architechture and carbon fixation (Jez et al., 2016). Hence, we quantified transcripts level in leaves and roots of maize seedlings inoculated to RZ2MS9 and Control-treated cultivated *in vitro* and under greenhouse conditions for eighteen maize genes: pathogenesis related 1 *(pr1),* lypoxidase *(lox1)*, respiratory burst oxidase protein C (*rbohC)*, *β*-glucosidase (*Bglu60.1),* arginine decarboxilase *(adc),* ornithine decarboxilase (*odc*), auxin transporter-like protein 1 (*aux1)*, beta expansin 18 (*expb18),* Aux-IAA gene 14 (*iaa14)*, cell wall invertases 1 and 4 (*incw1, incw4)* soluble invertase 1 and 2 *(ivr1, ivr2),* cis-zeatin glicosyl transferase (*cZgt),* giberellic acid stimulated-like 4 and 5 *(gsl4,gsl5), PEP carboxylase (pepc), NADP malic enzyme (me)*, plus maize reference genes tubulin beta (*β-tub*) and membrane protein PB1A10.07c coding gene (*mep*). Aditionally, RZ2MS9 bacterization interference in maize growth parameters such as chlorophyll, dry matter and soluble sugars content were quantified from plants grown under same conditions. At last, we quantified the expression *in vitro* and *in planta* of RZ2MS9 *miaA*, keystone microbial gene involved in bacterial stress response and cytokinin synthesis activated in benefic plant microbe interaction to investigate its possible contribution to observed chlorophyll content increase in leaves of seedlings interacting to RZ2MS9.

4.2. MATERIAL AND METHODS

4.2.1. Biological material

Bacillus thuringiensis RZ2MS9 was isolated from guarana (*Paullinea cupana*) rhizosphere at Maues-AM Brazil (Batista et al., 2018). RZ2MS9 and *Pseudomonas protegens* PF5 belong to Laboratory of Microrganisms Genetics “Prof. João Lúcio de Azevedo” microbes collection, Department of Genetics, University of São Paulo, Piracicaba-SP, Brazil. Commercial DiPel® (*Bacillus thuringiensis* serovar *kurstaki* HD1) was kindly supplied by professor Italo Delalibera Júnior, Pathology and Microbial Control Laboratory, Department of Entomology and Acarology, University of São Paulo, Piracicaba-SP, Brazil. Commercial Kodiak ® (*Bacillus subtilis* GBO3) was gently provided by professor Dr. José Mauricio Simões Bento, Laboratory of Chemical Ecology and Insect behavior, Department of Entomology and Acarology, University of São Paulo, Piracicaba-SP, Brazil.

Bacterial colonies were stored in 20% glycerol at -80°C and routinely cultivated in Luria-Bertani Agar at 28º C (Sambrook & Russell, 2001). Maize hybrid Pioneer ® P4285H (F1) seeds were kindly provided by professor Roberto Fritsche-Neto, Allogamous Laboratory, Genetics Department – University of São Paulo – USP/ESALQ Piracicaba-SP, Brazil.

4.2.2. Maize-RZ2MS9 gnotobiotic colonization assay

Pioneer ® P4285H maize seeds were surface disinfested at laminar flow chamber in following sterile solutions: 70% ethanol for 1 min, 2% sodium hypochlorite for 2 min, 70% ethanol for 1 min, and twice washed in sterile deionized water. Seeds were germinated and grown under axenic conditions for 6 days in Petri dishes (90mm) containing triple layered Germitest® paper disks soaked in 3 mL sterile deionized water. Contaminated Petri dishes were eliminated. Under sterile environment, 6 days-old germinated axenic maize seedlings were transfered to 50 mL Falcon® tubes containing 7 mL of Murashige-Skoog (MS) pH 6,0 with half of sucrose content to mimic rhizosphere oligotrophy (Ramos et al., 2000; Trigiano & Gray, 2010). The seedlings were positioned with roots tips soaked into MS and both seed and aerial parts were kept above the broth. RZ2MS9 cells (10^8 CFU/mL) grown in Luria Bertani broth for 9 h at 28 ºC (180 rpm) were directly added into MS broth. The falcons were kept half closed and wrapped in Parafilm® to allow only gas exchanges with external atmosphere for 7

days under 15 h of photophase. Control-treated and bacterized seedlings were collected 120 h.p.i. at V2 stage and immediately frozen in liquid nitrogen.

4.2.3. Maize-RZ2MS9 greenhouse colonization assay

Pioneer ® P4285H maize seeds were surface disinfested at laminar flow chamber in following sterile solutions: 70% ethanol for 1 min, 2% sodium hypochlorite for 2 min, 70% ethanol for 1 min, and twice washed in sterile deionized water. Seeds were instantly soaked into sucrose (10%) solution, and were kept for 30 s until solution drying. Bacterization was made by soaking the sucrose treated seeds into a suspension of RZ2MS9 cells (10^8 CFU/mL) for 30 min (Batista et al., 2018). RZ2MS9 inoculum was previously grown in Luria Bertani (LB) broth for 9 h at 28 ºC (180 rpm). Control treatment consisted in the addition of respective volume of LB broth equal to inoculum volume used for bacterization.

Maize seeds were germinated in 5 L plastic vases containing substrate BASAPLANT ® which contained minimal nutritional requirements. Plants were watered daily. Seedlings were harvested at V2 stage, 6 days after germination (d.a.g.) and immediately frozen in liquid nitrogen. Experiment design was entirely randomized consisted in two treatments, RZ2MS9 and Control, with thirty-six replicates of one maize plant each.

4.2.4. Growth parameters assessment

Eighteen maize seedlings cultivated *in vitro* and under greenhouse condition were randomly collected from RZ2MS9 and Control treatments at stage V2. Shoot and root fresh weight were immediately measured. Dry weight was measured after incubation of plant samples at 65ºC in paper bags for 4 days until reaching constant weight.

4.2.5. Total soluble sugars quantification

0.5 gram of freshly harvested leaves or roots of V2 stage bacterized and Control maize plants cultivated *in vitro* and under greenhouse were grinded in 5 mL of 95% (v/v) ethanol. Soluble fraction was collected and the insoluble fraction was then washed with 5 ml of 70% ethanol twice. Total obtained soluble fractions were centrifuged for 10 min at 3500 g. The supernatants were collected and stored at 4°C for total soluble sugars quantification. 0.1 mL of the alcoholic extract was reacted with 3 ml anthrone

(150 mg anthrone + 100 ml 72% [fw/w] H_2SO_4) freshly prepared and placed for 10 min in a boiling water bath. Samples were cooled and had absorbance taken at 625 nm in spectrophotometer Ultrospec 3000 Amersham Pharmacia Biotech, expressed in mg per gram of fresh weight (Irigoyen et al., 1992; Yadav et al., 2002).

4.2.6. Photossynthetic pigments extraction and quantification

Protocol was adapted from Hendry & Grime (1993). Ten 0.69 cm² leaf blade disks from RZ2MS9 bacterized and Control-treated V2 seedlings previously grown *in vitro* or under greenhouse conditions were sampled from expanded leaves using perforator. Seedlings were harvested and kept in a cold termical bag prior to leaf disks sampling. Leaf disks were soaked in 10 mL of acetone 80% (v/v) and kept in darkness at 4ºC for 72 h. Pigments solutions were agitated daily.

Absorbance of photosynthetic pigments was measured at 480, 645 and 663nm (Ultrospec 3000 Amersham Pharmacia Biotech) and used for pigment quantification using specific equations (Hendry & Grime, 1993). As a result, chlorophyll a, b and carotens levels were estimated in µmol per gram of fresh tissue weight.

4.2.7. Screening of microbial cytokinin production

Bacillus spp RZ2MS9, HD1 and GBO3 and *Pseudomonas protegens* Pf-5 strains were streaked on half of petri plate containing M9 medium amended with 0.2% Casamino Acids, 0.01% thiamine, and 2 ug of biotin per L (Akiyoshi et al., 1987). 50 µmol/mL kinetin solution was streaked in M9 plates as positive cytoninin control. *B. subtilis* GBO3 was used as a cytokinin producing bacterium (Mounde, 2014). *P. protegens* PF5 strain was used as non-producing cytokinin bacterium negative control. Sterile water was streaked in M9 plates as negative essay control. The plates were incubated at 28°C for 96 h. Four etiolated cucumber cotyledons were harvested of cucumber seedlings previously grown in the darkness in paper disks moisturezed with sterile water for six days were positioned 2 cm apart of M9 bacterial culture and control plates in the dark. The plates were kept in darkness for 20 h, covered with aluminium foil and then kept in light for 3h (Hussain & Hasnain, 2009; 2011). Cotyledons were immediately harvested and submitted to cold acetone chlorophyll extraction followed by taking absorbance at 663 nm (Ultrospec 3000 Amersham Pharmacia Biotech). Ten replicates were used per treatment, each one containing two cotyledons. Chrolophyll

content was measured as ratio (D/D0) between sampled content (D) and water negative control content (D0) (Hussain & Hasnain, 2011).

4.2.8. Statistical analysis

Maize seedlings fresh and dry weight data were submitted to Kruskal-Wallis test ($p<0.05$). Data analysis was performed using R studio package “Laercio”. Total soluble sugars from maize leaves and roots, and maize leaf photosynthetic pigments data analysis was executed one-way ANOVA with replicates using Microsoft Excel. Significance of difference among RZ2MS9 and Control treatment means was accessed considering calculated C.D. ($p<0.05$). Chlorophyll levels of cucumber cotyledons were processed using Duncan’s multiple range test.

4.2.9. Expression analysis

4.2.9.1. Maize gene expression during RZ2MS9 interaction

Two maize housekeeping genes with stable and constitutively described expression were used to normalize maize genes expression during RZ2MS9 interaction: tubulin beta (*β-TUB*) and membrane protein PB1A10.07c coding gene (*MEP*) (Manoli et al., 2012; Lin et al., 2014). All target genes and respective primers employed are shown in table 1.

Table 1 – List of primer sequence and sampled tissue used in qRT-PCR analysis for *Zea mays* transcript quantification *in vitro* and under greenhouse conditions with respective references.

***Zea mays* gene**	**Name**	**Tissue**	**Sequences**	**Reference**
Biotic interaction and defense related genes				
pr1	*pathogenesis related protein 1 (coded)*	leaves	TCAGTCATGCCGTTCAGCTT	Gond et al., 2015
		roots	TTGTCCGCGTCCAGGAA	
lox	*Lypoxidase*	leaves	AGGAGTTTGGACGGGAGATT	Ding et al., 2017
		roots	CCGTACTTGCTCGGGTCA	
bohc	*respirat burst oxidase protein C*	leaves	TTCTCTTGCCTGTATGCCGC	Zhang et al., 2010
		roots	CTTTCGTATTCCGCAGCCA	
bglu	*beta glucosidase*	leaves	GATTACATCCAGCGCCACATC	Vyroubalová et al., 2009
		roots	AAGCGAAGTAGCCTTGCACATT	
adc	*arginine decarboxilase*	leaves	CTATAACTACCACATGAACCTCT	Rodríguez-Kessler et al., 2009
		roots	GTACGGCCACGTAGTAGCCAC	
odc	*ornithine decarboxilase*	leaves	TGTGCTAGCCGTGCTGAAATT	Rodríguez -Kessler et al., 2006
		roots	TTGCTGCGGTGGTGAACTGGTG	
growth and sucrose metabolism related genes				
iaa14	*Aux-IAA*	leaves	AGATGTTGCCCATTGTATCAGAA	Ludwig et al., 2013
		roots	GGAGACACGGTAGGGGACA	
aux1	*Auxin transporter-like protein 1*	leaves	CGAAACGCACCCTGCATT	Plucani do Amaral et al., 2014
		roots	CCCGCTTTTTACAGTGGAAGAT	
expb18	*expansin beta*	leaves	ATGCAGAACAACTCCGGGTA	Muller et al., 2007
		roots	TACTGGACGAAGGAGCGGTA	
gsl4	*Gibberellic Acid Stimulated-Like*	roots	CGAGAAACCTCCAGTCCAGC GGTCGGATCATACAAAACGTG	Zimmermann et al., 2010
gsl5	*Gibberellic Acid Stimulated-Like*	leaves	TCCATCCACCGTGGTTCCCAG GTTTTAGACATGACCACTGTATTG	
czogt	*Cis-zeatin glicosyl transferase*	leaves	CAGGCCCTGGGAGAAACA	Vyroubalová et al., 2009
		roots	AGCATTGCCTCCTCGATCAC	
ivr1	*soluble invertase 1*	leaves	TGGTGGAGGTGGAGAAC	Behr et al, 2010
		roots	GAACACAGCCTCGATGTC	
ivr2	*soluble invertase 2*	leaves	GCGTCCAAGACGTTCTAC	
		roots	TACCCGTCTTGGTGTCC	
incw1	*cell wall invertase 1*	leaves	GTCTTCTTCAGGGTCTTCAG	
		roots	GTCGGCTTGTACAGATCC	
incw4	*cell wall invertase 2*	leaves	TTGAGGAGCACGAGACC	
		roots	TACACTCGAGCCGTGATG	
sh1	*shrunken1 - sucrose synthase*	leaves	GGAGTAGCCTGCGTTCTACG	Shu et al., 2015
		roots	GTCAATGTGCAGGCCAGATA	
Photossynthesis Related Genes				
me	*NAPD malic enzyme*	leaves	GATCTCTGCGCACATCGCTGC GCAGCACTACCGGTAGTTGCGG	Hahnen et al., 2003
pepc	*PEP carboxilase*	leaves	AGAACTCAAGCCCTTTGGGAAGC	

			GTCGGCGAACTCCTTGGACAGC	
		Maize Reference Genes		
β-tub	*tubulin beta*	leaves roots	CTACCTCACGGCATCTGCTATGT GTCACACACACTCGACTTCACG	Lin et al., 2014
mep	*membrane protein PB1A10.07c*	leaves roots	TGTACTCGGCAATGCTCTTG TTTGATGCTCCAGGCTTACC	Manoli et al., 2012

4.2.9.2. RZ2MS9 relative gene expression *in vitro* and during maize interaction

The relative expression of gene *miaA* from RZ2MS9 was calculated considering efficency (E) and Cq values from raw amplification data and LinReg via a modified Pfaffl equation (Hellemans et al. 2007; Pfaffl 2001) that included in the denominator the geometric mean of reference genes (RG). Normalized Relative Quantity (NRQ) equation described by Smith et al (2018) is presented below:

$$NRQ = \frac{E_{GOI}^{(Cq_{control} - Cq_{treatment})^{GOI}}}{\prod E_{RG}^{(Cq_{control} - Cq_{treatment})^{RG}}}$$

The refered NRQ equation precognized the use of RT-qPCR data from the *in vitro* culture of the bacterium of interest as template to obtain Cq reference values (named “Cq control” in equation above). Then, Cq values obtained for different treatments would be subtracted from the Cq reference values. In this work, the Cq reference values were obtained using RZ2MS9 culture in a specific medium that favored cytokinin production as template in RT-qPCR. This medium was M9 broth amended with 0.2% Casamino Acids, 0.01% thiamine, and 2 ug of biotin per liter (Akiyoshi et al., 1987) for 48h 150rpm at 28ºC (stationary growth phase, Batista, 2012). RZ2MS9 colonies were immediately treated with RNAprotect Bacteria Reagent ® (QIAGEN) prior to RNA extraction. The Cq values from treatments were obtained using cDNA from the same V2 stage maize seedlings bacterized with RZ2MS9 cultivated *in vitro* or under greenhouse conditions employed for maize gene quantification. Three *Bacillus* housekeeping genes with stable and constitutively described expression were used to normalize RZ2MS9 gene expression: *udp*, *fabI* and *rpoB* (Teixeira, 2017). Genes, primers sequences, inoculated tissue evaluated and references were displayed in table 2.

Table 2 – List of primer sequence and sampled tissue used in qRT-PCR analysis for RZ2MS9 gene transcript quantification. Primers were designed to target microbial cytokinin biosynthetic gene *miaA* transcripts from RZ2MS9 (OGY05594.1) in M9 medium specific for cytokinin production and in leaves and roots of maize seedlings bacterized with RZ2MS9. Asterisks represented two maize cultivation systems: *in vitro* (gnotobiotic) and greenhouse conditions. Three housekeeping genes employed for normalization of expression were also displayed (Teixeira, 2017).

RZ2MS9 genes	Name	Tissue/ medium analyzed	Sequences	Reference
Cytokinin biosynthetic gene				
miaA	*tRNA (adenosine(37)-N6)-dimethylallyltransferase*	Leaves* Roots* M9 broth	ATTCCACACCGCGTTCTAAA TTCAAGAACGTGTTCGCAAG	This work
Bacillus reference genes				
Udp	*UDP-N-acetylglucosamine 2-epimerase*	Leaves* Roots*	CATACCCAGAAGAGATGAAT CTGTAAGTTCGTTGCTGAT	Teixeira, 2017
fabI	*Enoyl reductase I*	Leaves* Roots*	CATTAGAAGGACAAGAATCA GTGCTAGACCGTGAATAGTA	Teixeira, 2017
rpoB	*DNA-directed RNA polymerase subunit beta*	Leaves* Roots*	AGACAACACAGATAGCACAG GAAGAAACGAGACACAAGTA	Teixeira, 2017

4.2.9.3. Primer Designing

Primer 3 software version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/) was used to design specific set of primers from nucleotide sequences of RZ2MS9 *miaA* gene for Reverse Transcription Polymerase Chain Reaction quantitative real time (RT-qPCR). Primer3 and Oligo Analysis Tool software were employed for hairpins and dimers detection during design step (http://www.operon.com/tools/oligo-analysistool). Additionally, primers sequences specificity was tested using NCBI BLASTn tool and Plant Comparative Genomics database Phytozome ® v 12.1 BLAST using deposited maize genomes.

4.2.9.4. Conventional Polymerase Chain Reaction (cPCR)

Primers specificity to maize template was verified by Polymerase Chain Reactions (PCR) made with the following templates: Pioneer P4285H aseptically cultivated maize seedlings DNA and *Bacillus* sp. RZ2MS9 DNA as negative control. Bacterial DNA was extracted using DNeasy ® Blood & Tissue Kit (QIAGEN). DNA of sterile cultured maize seedlings was extracted using DNeasy ® Mini Plant Kit (QIAGEN) as recommended by the manufacturer.

PCR reactions were optimized in Profex® PCR System thermal cycler (Applied Biosystems) in 25 µL reactions: 1x Taq DNA Buffer, 3.5 mM MgCl2, 0.2 mM DNTP, 5 pmol of each primer (forward and reverse), 1.25 U of Taq DNA polymerase (Thermo Scientific), 50 ng of template DNA and Milli-Q water (qsp). The PCR cycles consisted in initial denaturation step at 95 °C for 2 min, 35 denaturation cycles at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s, and final extension step at 72 °C for 10 min. PCR amplification products were quantified in BioDrop DUO® In-built Sample Port and electrophoresed in 2% agarose gel, purified with Illustra GFX ® PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequenced by Human Genome and Stem Cells Research Center University of São Paulo, São Paulo – SP, Brazil. Specificity was confirmed by comparison of retrieved amplified sequences and the original maize coding ones using the MEGA 6.0 (Tamura et al., 2013).

4.2.9.5. Reverse Transcription Polymerase Chain Reaction quantitative real time (RT-qPCR)

Frozen maize seedlings cultivated *in vitro* and under greenhouse conditions were ground into a fine frozen powder using nuclease-free mortar, pestle and liquid nitrogen. Total RNA was extracted with the RNeasy Plant Mini Kit (QIAGEN) using manufacturer's conditions and RNase-free DNase treatment in-column (QIAGEN). Concentration and quality of total RNA were measured in BioDrop DUO® In-built Sample Port and 1% agarose gel electrophoresis. Total bacterial RNA from RZ2MS9 M9 broth culture was extracted with the RNeasy Plus Mini Kit (QIAGEN) using manufacturer's conditions and RNase-free DNase treatment in-column (QIAGEN). The cDNA synthesis was done using RevertAid® H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) in Profex® PCR System thermal cycler (Applied Biosystems). The reactions were performed according to the manufacturer's conditions: 4 µg of total RNA, 5 pmol of random primer, 4 µL of Reaction Buffer (5X), 1 mM of dNTP mix, 20 U of RiboLock® RNase Inhibitor, 200 U of RevertAid H Minus Reverse Transcriptase and RNase-free water qsp in a final volume of 20 µL.

The qPCR reactions were performed in an Applied Biosystems 7300 Real-Time PCR System in using GoTaq® qPCR System (Promega). Technical duplicates of the four biological replicates were employed for *in vitro* and greenhouse cultivated maize seedlings for RZ2MS9 treatment, control treatment and RZ2MS9 cultured in M9 broth (Bustin et al., 2009). Each reaction consisted of 6.25 µL GoTaq® master-mix, 3.625

µL nuclease-free water, 0.125 µL CXR Reference Dye, 10 pmol of each primer and 2 µL of template cDNA (250 ng/µL), into a final volume of 12.5 µL. The qPCR parameters were 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 58 °C for 45 s, and standard equipment dissociation stage for melting curve.

4.2.9.6. Statistical analysis of gene expression

The efficiency of maize and RZ2MS9 gene expression was calculated by LinRegPCR v.11.0. Relative expression data of maize genes was calculated by PFAFFL method using Relative Expression Software Tool (REST) (Pfaffl, Horgan & Dempfle, 2002). Relative expression of RZ2MS9 genes was calculated using NRQ equation (Smith et al., 2018) Differential expression for maize and RZ2MS9 genes was checked by Pairwise Fixed reallocation Randomization Test (2000 bootstraps).

4.3. RESULTS

4.3.1. Growth parameters and total soluble sugars quantification

Fresh matter, dry matter and total soluble sugars content were quantified for leaves and roots of V2 stage maize P4285H seedlings bacterized with RZ2MS9 cultivated *in vitro* and under greenhouse conditions comparing to Control-treated seedlings. RZ2MS9 treated plants had higher root fresh and dry weight content for both cultivation systems: *in vitro* and greenhouse (Figure 1).

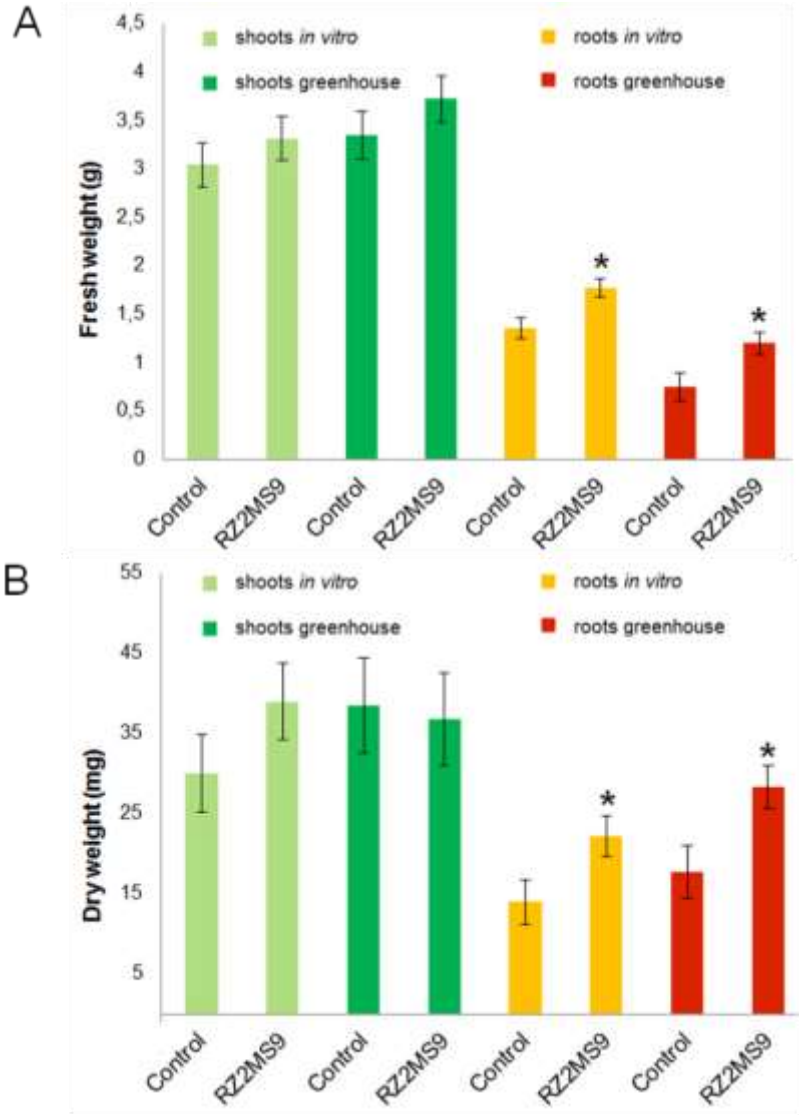


Figure 1 – Fresh and dry weight quantification across shoots and roots of V2 stage maize P4285H seedlings cultivated *in vitro* and under greenhouse conditions RZ2MS9 and Control. Fresh weight (A) and dry weight (B) data were presented, respectively, in grams and in milligrams per shoot or root of single plants. Eighteen repetitions were employed, and significant differences were shown with an asterisk, according to Kruskal-Wallis test ($p<0.05$).

Total soluble sugar content was significantly increased in leaves and roots of RZ2MS9 treatment under greenhouse conditions at 5% significance (Figure 2).

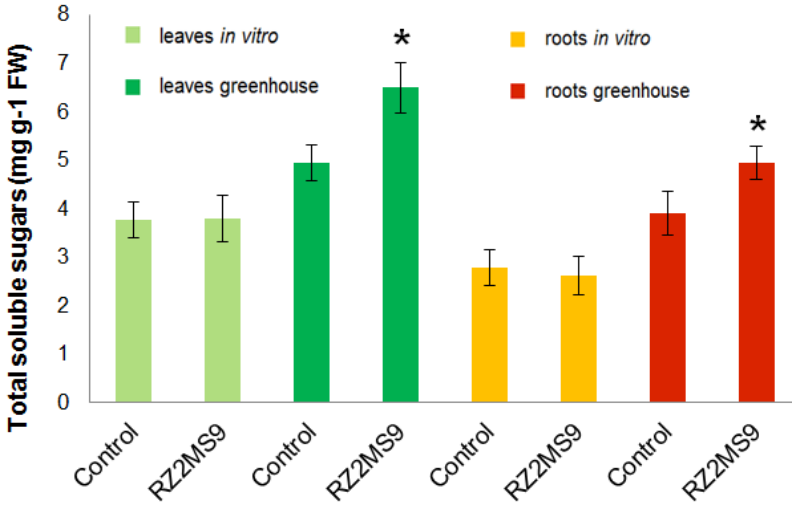


Figure 2 – Total soluble sugar quantification in leaves and roots of V2 stage maize P4285H seedlings bacterized with RZ2MS9 and Control-treated cultivated *in vitro* and under greenhouse conditions. Measurement was in miligrams per gram of fresh weight. Data analysis was executed one-way ANOVA with replicates (Microsoft Excel). Significance of difference among RZ2MS9 and Control treatment means was accessed considering calculated C.D. ($p<0.05$).

4.3.2. Photossynthetic pigments quantification

RZ2MS9 and Control treated maize seedlings cultivated *in vitro* and under greenhouse conditions had their content of photosynthetic pigments estimated. Remarkbly, levels of chrolophyll a and chlorophyll b were significantly increased in RZ2MS9 treatment comparing to Control in greenhouse cultivation (Figure 3).

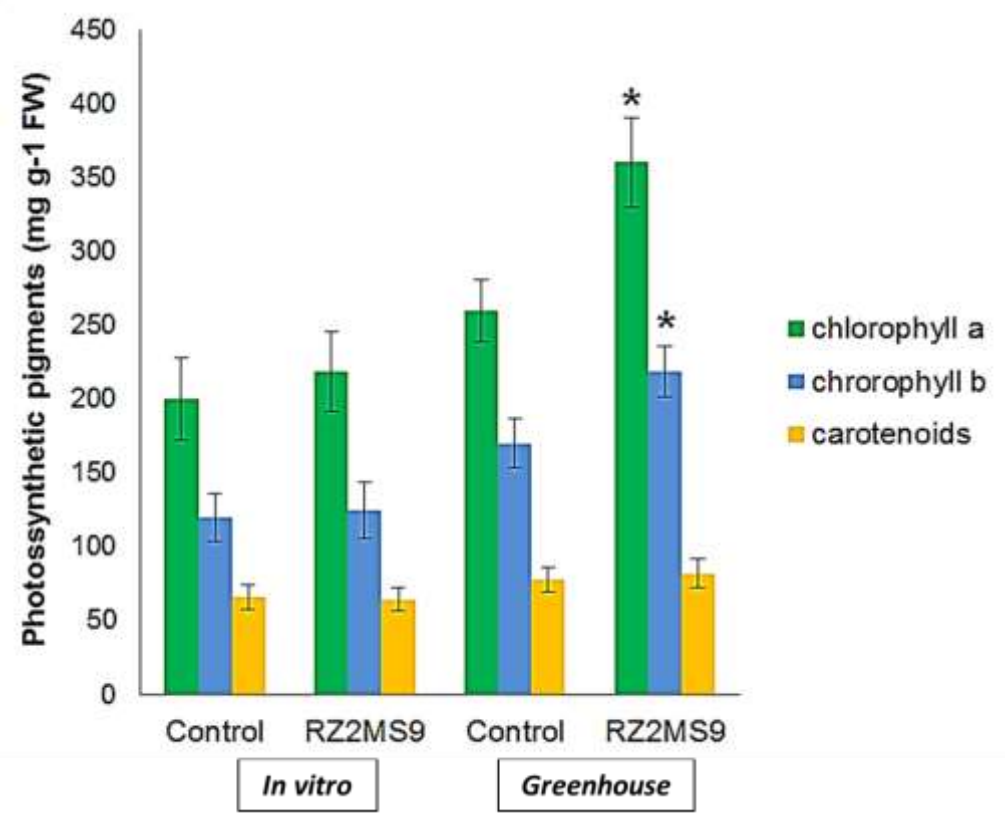
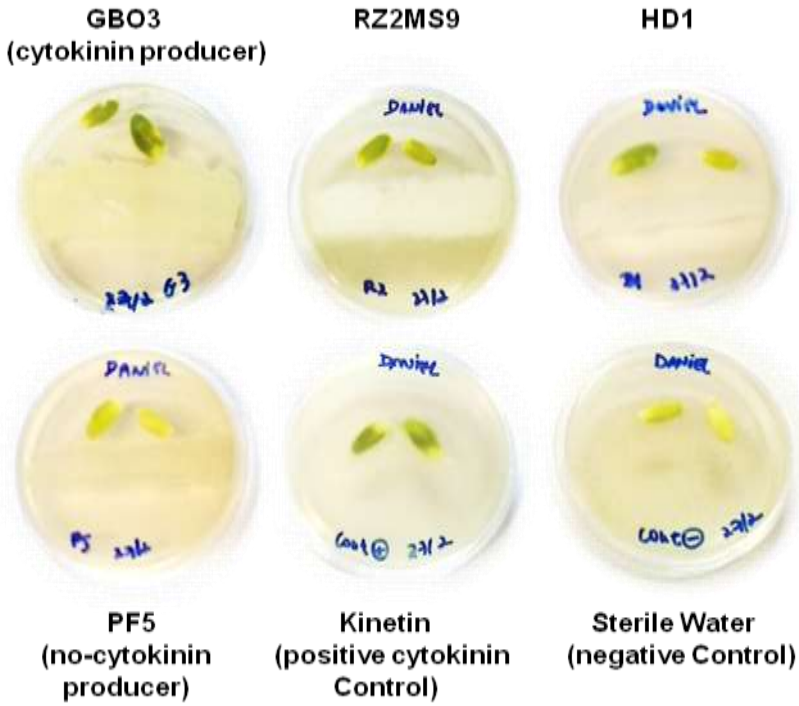


Figure 3 – Quantification of photossynthethic pigments of leaves of V2 stage maize P4285H seedlings cultivated *in vitro* and under greenhouse conditions RZ2MS9 and Control treated extracted by cold acetone and measured in spectrophotometry. Data analysis was executed one-way ANOVA with replicates (Microsoft Excel). Significance of difference among RZ2MS9 and Control treatment means was accessed considering calculated C.D. ($p<0.05$).

4.3.3. Screening of microbial cytokinin production

RZ2MS9 and cytokinin producing strain GBO3 stimulated chlorophyll levels in cucumber cotyledons similarly to control kinetin (50 µmol) as shown in figure 5. As a negative control non-producing cytoninin strain PF5 did not differed from water control. HD1 strain showed an intermediary result comparing to other *Bacillus* strains and control.



Strains	*D/D0*
Bacillus subtilis GBO3 (cytokinin producer)	1.480 (fg)
Bacillus thuringiensis RZ2MS9	1.440 (fg)
Bacillus thuringiensis serovar. *kustakie* HD1	1.340 (cde)
Pseudomonas protegens PF5 (non-cytokinin producer)	1.090 (ab)
Sterile water (negative control – reference)	1.000 (a)
Kinetin 50 µmol (positive control)	1.485 (fg)

Figure 4 – Screening of microbial cytokinin production by cucumber cotyledon essay (above) and relative quantification of photossynthethic pigments of etiolated cucumber cotyledons extracted by cold acetone and measured in spectrophotometry. Data was processed with Duncan's multiple range test, and significant differences between RZ2MS9 and Control treatments were displayed with different letters.

4.3.4. Reverse Transcription Polymerase Chain Reaction quantitative real time (RT-qPCR)

Fourteen maize genes had their expression changed by RZ2MS9 treatment under cultivation conditions as detected by RT-qPCR (Figures 5, 6 and 7).

Considering biotic interaction and defense related maize genes, maize salicylic acid responsive gene *pr1 (pathogenesis related protein 1),* jasmonic acid responsive gene *lox (lypoxidase)* and *bglu60.1 (β-glucosidase)* were approximately 2-fold up-expressed only in leaves of RZ2MS9 treated maize seedlings cultivated *in vitro* and under greenhouse conditions (Figure 5). In roots of inoculated maize seedlings cultivated *in vitro*, *adc* (arginine decarboxylase) and *odc* (ornithine decarboxylase) genes were 2.5-fold significantly up-expressed comparing to control plants.

Remarkbly, *adc* and *odc* were 21-fold and 2-fold up-expressed in maize roots of RZ2MS9 bacterized seedlings cultivated under greenhouse conditions (Figure 5).

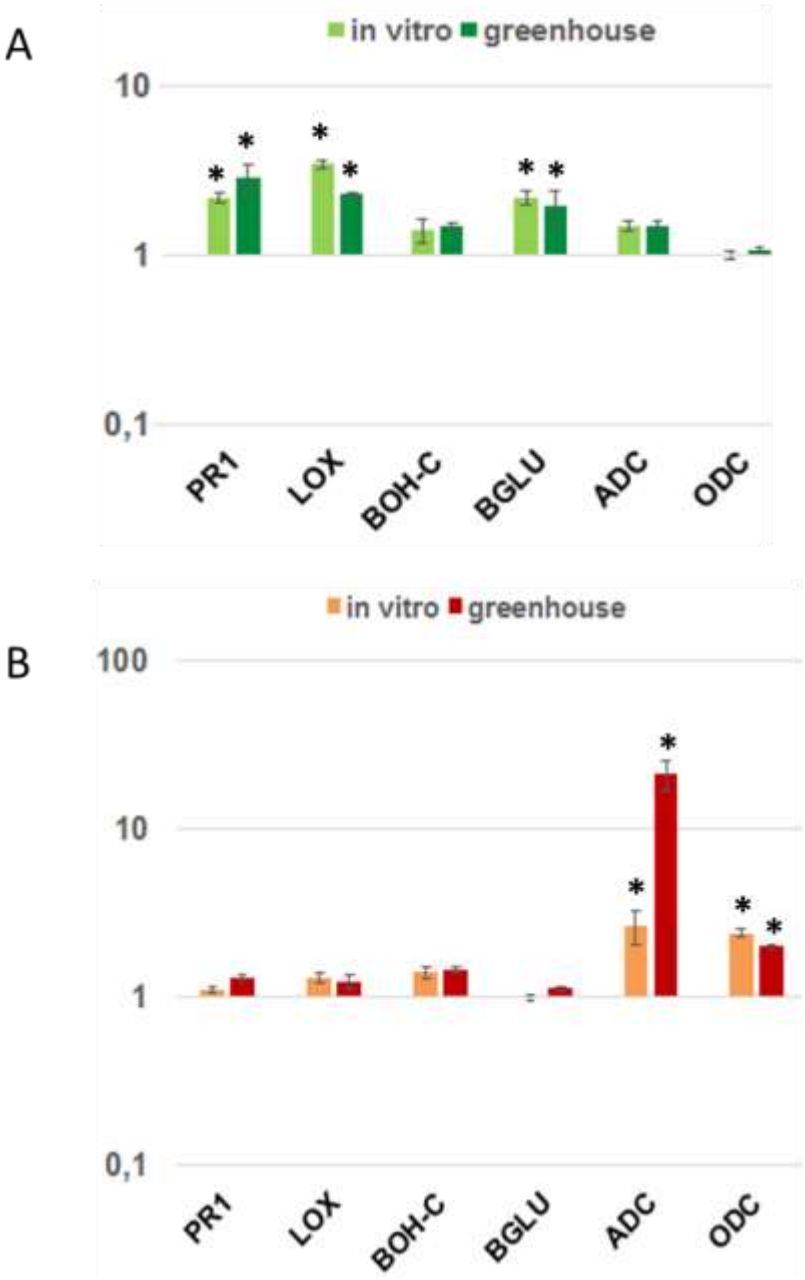


Figure 5 – Relative expression data of biotic interaction and defense related maize genes calculated by PFAFFL method of Control-treated maize P4285H seedlings comparing to RZ2MS9 treatment *in vitro* and under greenhouse conditions. Transcripts were named according to coding gene. A: Results obtained using leaf tissue sampled of V2 stage maize seedlings. B: Results obtained using root tissue sampled of V2 stage maize seedlings. Maize genes with significant differential expression ($p<0.05$) comparing to normalizing genes checked by pairwise fixed reallocation randomization test (2000 bootstraps) were spotted with an asterisk. Standard error was calculated considering three biological and two technical replicates.

Relative expression of growth and photossynthesis related maize genes were presented in figure 6. *Gsl5* gene was down-expressed only in leaves of bacterized seedlings *in vitro*. In roots samples tissues only *iaa14* gene was 2.5 and 2-fold up-expressed *in vitro* and under greenhouse cultivation conditions in RZ2MS9 treated maize seedlings (Figure 6). Cis-zeatin glicosyl transferase coding gene was down-expressed in both analyzed conditions for root samples comparing to Control treatment. EXPB18 (expansin beta 18) coding gene was only down-expressed in roots of bacterized maize seedlings cultivated under greenhouse conditions comparing to control. Two photossynthetic marker genes were down-expressed (*me1, pep1*) *in vitro* and under greenhouse cultivation conditions due to RZ2MS9 presence (Figure 6).

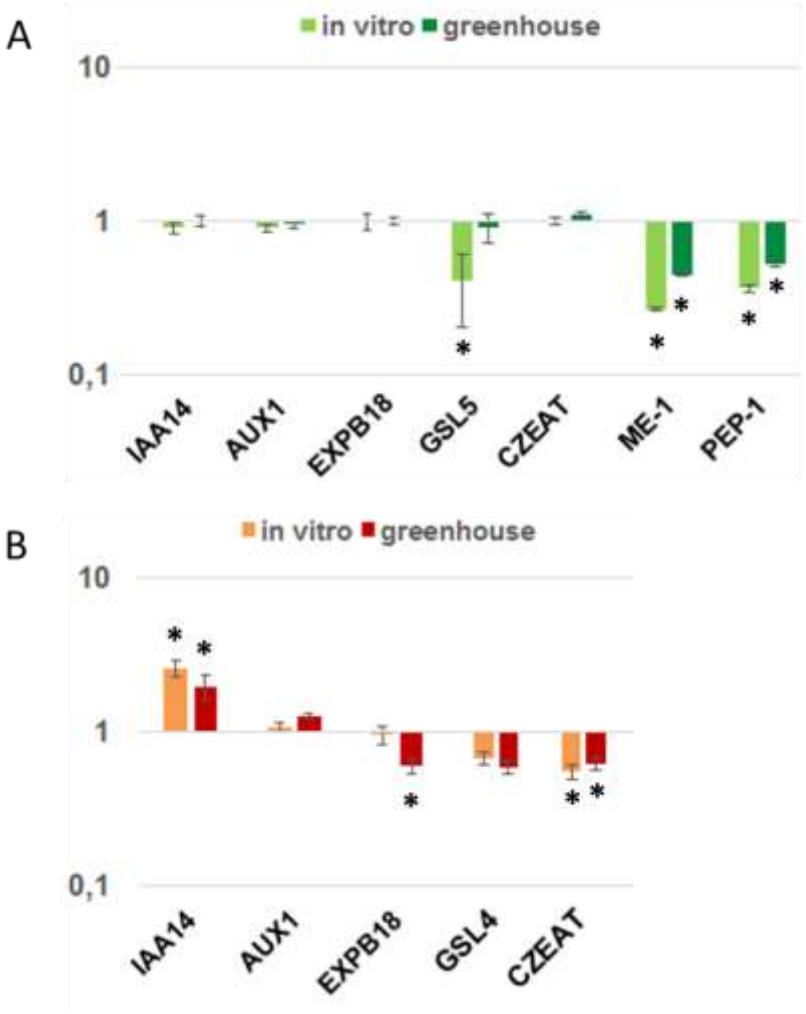


Figure 6 – Maize genes Relative expression data of growth and photossynthesis related maize genes calculated by PFAFFL method of Control-treated maize P4285H seedlings comparing to RZ2MS9 treatment *in vitro* and under greenhouse conditions. Transcripts were named according to coding gene. 1A: Results obtained using leaf tissue sampled of V2 stage maize seedlings. 1B: Results obtained using root tissue sampled of V2 stage maize seedlings. Maize genes with significant differential expression ($p<0.05$) comparing to normalizing genes checked by pairwise fixed reallocation randomization test (2000 bootstraps) were spotted with an asterisk. Standard error was calculated considering three biological and two technical replicates.

As for sucrose metabolism maize genes, *vac2 (vacuolar invertase 2)* and *susy (sucrose synthase)* were up-regulated approximately 6-fold and 4-fold respectively in maize roots and leaves comparing to control *in vitro* and under greenhouse conditions. Comparing to control *Vac1* gene was down-expressed *in vitro* and *wall4* was up-expressed in maize roots only under greenhouse conditions. Cell wall invertase 4 coding gene (*inv4)* was up-expressed only in roots of bacterized plants cultivated *in vitro* (Figure 7).

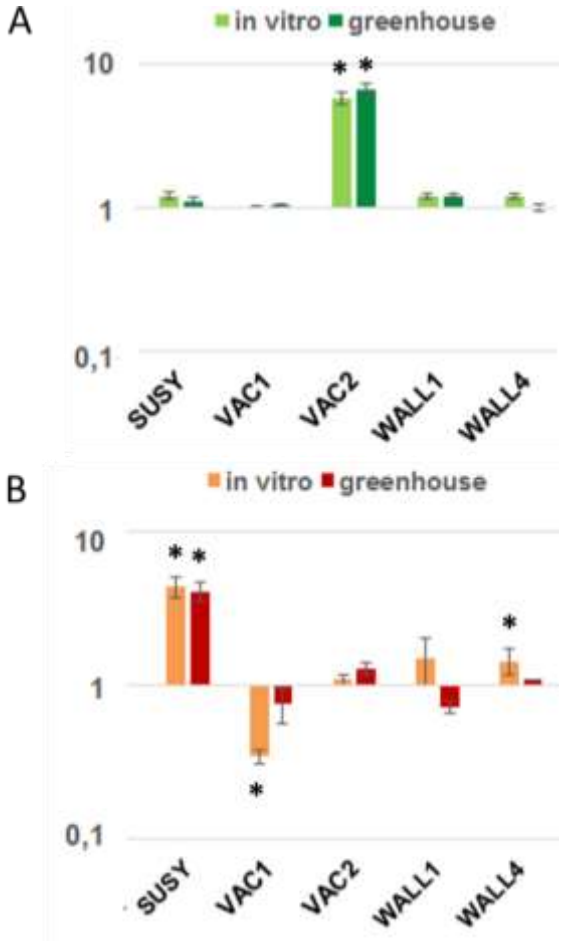


Figure 7 – Sucrose metabolism maize genes relative expression data calculated by PFAFFL method of Control-treated maize P4285H seedlings comparing to RZ2MS9 treatment *in vitro* and under greenhouse conditions. Transcripts were named according to coding gene. 2A: Results obtained using leaf tissue sampled of V2 stage maize seedlings. 2B: Results obtained using root tissue sampled of V2 stage maize seedlings. Maize genes with significant differential expression ($p<0.05$) comparing to normalizing genes checked by pairwise fixed reallocation randomization test (1000 bootstraps) were spotted with an asterisk. Standard error was calculated considering three biological and two technical replicates.

Only three target maize genes had any transcriptional change under the same conditions: *bohC, aux1, wall1* (Figures 5-7). Interestingly, the majority of analyzed genes (94.7% for sampled leaves and 75% for sampled roots tissues) kept expression pattern comparing the two cultivation methods analyzed: *in vitro* and greenhouse.

4.3.5. Quantification of RZ2MS9 *miaA* transcripts

RZ2MS9 relative *miaA* gene expression results expressed as Normalized Relative Quantity were shown in figure 5. *MiaA* gene from RZ2MS9 was down-expressed in leaves and roots of bacterized maize seedlings cultivated *in vitro* (gnotobiotic conditions). Remarkably, *miaA* gene expression levels quantified in leaves and roots of bacterized maize seedlings cultivated under greenhouse condition did not

differ from *miaA* expression levels measured from M9 broth, under favoring cytokinin production conditions. *MiaA* gene was up-expressed 2-fold and 2.5-fold respectively in leaves and roots of maize bacterized seedlings cultivated in greenhouse comparing to leaves and roots of maize bacterized seedlings cultivated *in vitro* (Figure 8).

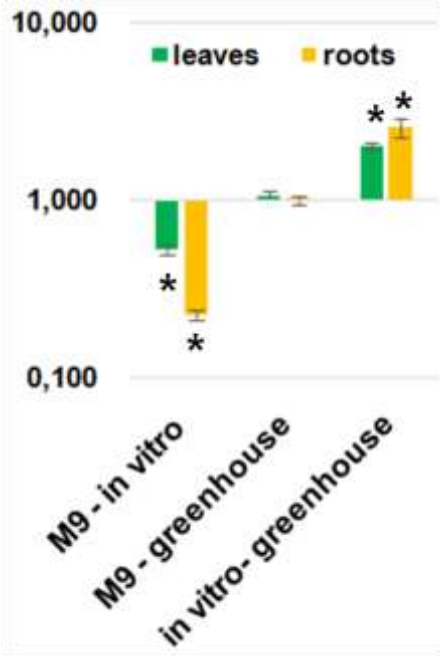


Figure 8 – Relative expression of *miaA* gene from across diferent treatments: M9 broth, leaves and roots of bacterized maize seedlings harvested at V2 stage cultivated in vitro and in greenhouse. M9 broth favored cytokinin production *in vitro* corroborated by enhanced chlorophyll content in cucumber cotyledon essay. RZ2MS9 culture in M9 was used as reference Cq values to perform relative expression comparing to Cq obtained from two maize interaction treatments using NRQ: *in vitro* (gnotobiotic) and cultivated under greenhouse conditions. Relative expression was also calculated comparing *in vitro* (gnotobiotic) and cultivated under greenhouse conditions. Significant differential expression ($p<0.05$) comparing to control was checked by pairwise fixed reallocation randomization test (2000 bootstraps) were spotted with an asterisk. Standard error was calculated considering three biological and two technical replicates.

4.4. DISCUSSION

Plant inoculation with beneficial microorganisms for instance Plant Growth Promoting Rhizobacteria (PGPR), has developed a promising approach to increase crop yield (Azevedo et al, 2000; Jha et al., 2013; Dey et al., 2014; Goswami et al, 2016). Roots constitute prime interaction site of plant-microbe relationship which exerce crucial role in plant physiology as primary sink organ major pool of cytokinins production (Ramos et al., 2000; Trigiano & Gray, 2010). For annual crops such as maize and soybeans, increases in root sink capacity during early vegetative growth phase might increase final stand performance due to better soil exploration and resurces extraction by the plant (Lanna-Filho et al., 2010). As consequence,

enrichment of water and nutritional status sustain higher sources demands and photossynthethic capacity.

Based on the relevance of RZ2MS9 to maize growth promotion and changes in root architecture (Batista 2017; Batista et al., 2018) and considering scarcity of studies addressing plant response to PGPR inoculation at transcriptional level (Plucani do Amaral et al., 2014; Gond et al., 2015; Ding et al., 2017; Espindula et al., 2017), we directed our study toward investigation of maize genes modulated by RZ2MS9 and phenotypic changes and their related with growth parameters both *in vitro* and under greenhouse conditions.

Considering the first group of analyzed maize genes related with biotic interaction, including plant immune response markers (Verhage et al. 2010; Plucani do Amaral., 2014), these results are consistent to 2 to 5-fold up-expression of *pr1* and *lox* in maize seedlings inoculated with different *Bacillus* spp strains including *B. subtilis* (Gond et al., 2015; Ding et al., 2017). *β*-glucosidase activity has been related to multiple roles including glycoconjugates hydrolysis of hormones and toxic bioactive molecules important in defense response against herbivores and phytopathogens (Poulton et al., 1990; Phillips & Streit, 1996; Kittur et al., 2007; Stein & Granot, 2019). The release of plant hormones from conjugated inactive into free active forms constituted a rapid mechanism to mobilizate hormone to trigger transduction cascades than *de novo* biosynthesis which could reduce temporal window of host responses to stress conditions (Morant et al., 2008). This suggests RZ2MS9 could benefit plant response against herbivors beyond entomopatogenic potential described for this bacterium in chapter 1. Moreover, it constitutes the first description of plant *β*-glucosidase expression modulation by a benefical PGPR strain, as other studies focused on microbial *β*-glucosidase activity or gene expression (Glick et al., 2012, Dinesh et al., 2013). A single study evaluated maize and common beans *β*-glucosidase activity during inoculation of *Azospirillum brasilense* (Vedder-Weiss et al., 1999). *RbohC* expression did not change by RZ2MS9 presence, differently of up-expression described during maize-*Herbaspirillum seropedicae* interaction (Plucani do Amaral et al., 2014). Trifoliate orange *adc* gene overexpression notably enchanced plant resistance against ulcer disease (Wang, 2009). Likewise, ADC and ODC activities increase putrescine levels which contributed to higher resistance against insects in Chinese cabbage (Wang, 2007). Overall, afore mentioned gene expression results

suggested maize host defense activation due to host recognition of RZ2MS9 that might contribute to plant response to insect pests.

Maize cell wall invertases (CWI) and soluble invertases (SI) were connected, respectively to plant acid growth response, organs development, and sink strength, and had their coding genes up-expressed during plant biotic interaction (Tauzin & Giardina, 2014). Sink strength by definition is a plant organ capacity to retrieve photoassimilates, which depends on physical sink organ size and biochemically upon sink organ activity. Thus, sink strength measument mainly depends on net weight gain (Ho, 1988; Stein & Granot, 2019). Activation of genes coding for the afore mentioned sucrose catabolism enzymes in leaves and roots of maize due to RZ2MS9 presence associated to higher soluble sugar content under greenhouse cultivation suggests reduction of sucrose content available in the phloem. Consequently, natural process of sucrose phloem loading from source tissues might be stimulated, as an osmotic compensatory effect, harnessing maize roots sink strenght compared to Control plants. As none invertase or sucrose synthase coding genes were found in RZ2MS9 *draft* genome, the higher level of hexoses during maize-RZ2MS9 interaction have inhibited photosynthetic enzymes at V2 stage may have been directly consumed by the bacteria or plant. Noteworthy, both increase of total soluble sugars and up-expression of sucrose cleaving host enzymes were observed in RZ2MS9 bacterized plants. As glucose and sucrose increase were reported to down-regulate genes coding for photosynthetic enzymes (Krapp et al., 1993, Van Oosten & Besford, 1994, Pego et al., 2000), the higher soluble sugar content observed in leaves and roots of RZ2MS9 treated seedlings cultivated in greenhouse could have interfered in down-expression of photossynthetic marker genes *me1, pep1* quantified *in vitro* and under greenhouse cultivation conditions due to RZ2MS9 maize inoculation.

Considering growth-related maize genes, Aux-IAA gene *iaa14* is a key regulator in plant growth and development, particularly in auxin-regulated root branching and formation of adventitious roots (Fukaki et al., 2002), B-EXPANSIN 18 coding gene (*expb18*) expression was connected to auxin mediated cell enlargement in tissues with high rate of cellular division (Ludwig et al., 2013; Muller et al., 2007). In our study, *iaa14* was up-expressed *in vitro* and under greenhouse cultivation conditions *in root* tissues of RZ2MS9 treated maize seedlings comparing to Control and *expb18* was down-expressed for root samples in greenhouse comparing to Control treatment. As RZ2MS9 treatment increased adventitious roots formation and dry matter content of

V2 stage maize roots it was suggested that the bacterium stimulated higher host roots branching without resulting in cell size increase. This augmentation in absorption network might have contributed to enhanced sink activity in roots of RZ2MS9 treated greenhouse cultivated seedlings due to increased levels of transcripts of gene marker of sink strength *susy* (sucrose synthase) (Stein & Granot, 2019). Overall, these data suggest contribution of auxin responses during RZ2MS9 interaction in maize root system in specific way favoring phytostimulation of roots. RZ2MS9 benefic effect to maize root growth promotion was also described as 75.4% and 247.8% increase of root system dry weight in bcaterizated cultivar Altavista comparing to control under greenhouse cultivation (Batista et al., 2018).

In other studies, root growth of maize seedlings interacting to PGPR *Azospirillum lipoferum* was connected to increased gibberellin status also detected during interaction. This hypothesis was corroborated by observation of similar root growth stimulation in maize seedlings treated with GA3 giberellin comparing to *A. lipoferum* bacterization (Bottini, Cassán & Piccoli, 2004; Santi et al. 2013). However, in RZ2MS9 treated plants, root-specific *gsl4 (giberellin stimulated-like4)* was down-expressed in maize roots under greenhouse conditions, and shoot-especific *gsl5 (giberellin stimulated-like5)* was down-expressed in leaves of RZ2MS9 bacterizated seedlings. Apparently, RZ2MS9 presence might repress gibberellin responsive genes through an unknown mechanism.

Other significant phenotypic changes were observed after RZ2MS9 treatment to maize seedlings. Remarkbly, clorophyll content of leaves of bacterized maize seedlings greenhouse cultivated was higher than control. Moreover, cis-zeatin glicosyl transferase coding gene was down-expressed in roots of RZ2MS9 treated plants cultivated *in vitro* and in greenhouse. Photossynthetic pigment synthesis stimulation by cytokinin is documented (Chernyad'ev, 1993; Pospisilova et al., 1998). As cis-zeatin glicosyl transferase (*cZGT)* activity was related to inactivation of maize major cytokinin cis-zeatin (Veach et al., 2003; Vyroubalová et al., 2009), its gene repression observed in RZ2MS9 treatment might contributed to decrease conjugation of active zeatin into inactive glicosilated form at highest cytokinin producing site, the roots, subject to xylem transport into shoots. Aditionally, described up-expression of maize *β-glucosidase bglu60.1* gene in leaves after RZ2MS9 treatment *in vitro* and under greenhouse system might contribute to release of conjugated zeatin at chlorophyll synthesizing sites. Overall, RZ2MS9 contributed to increase in host leaves chlorophyll levels possibly

associated to cytokinin stimulation in which aforementioned *czgt* and *β-glu* might benefit due to increase amount of free hormone forms. Alternatively, the previoulsty described down-regulation itself of genes *me1, pep1* involved in C4 photossynthesis might have stimulated higher chlorophyll production in RZ2MS9 treated plants cultived in greenhouse as a compensatory effect that need further investigation.

Higher chlorophyll content described in wheat plants treated with cytokinin producing bacteria, comparable to chlorophyll levels reached by use of artificial cytokinin BAP (Arkhipova et al., 2006) and by the fact non-producing growth promoting bacteria evaluated at the afore mentioned study were not capable to increase chlorophyll levels nor increase cytokinin level of leaves of bacterizated plants (Arkhipova et al., 2006). In *Methylobacterium* spp epiphytic bacteria the turnover of isopentenylated tRNA production by bacterium than *de novo* synthesis of this phytormone (Koenig et al., 2002). Hence, we investigated if RZ2MS9 synthesis of cytokinin could have contributed to increase in maize leaves chlorophyll levels from RZ2MS9 bacterized seedlings cultivated in greenhouse.

In fact, *in vitro* production of cytokinin by RZ2MS9 was indirectly evidenced by increase in chlorophyl content in etyolated cucumber cotyledons comparable to chlorophyll levels obtained by using *B. subtilis* GBO3, a cytokinin producing bacterium and by cytokinin kinetin (Figure 4). Aditionally, a cytokinin non-producing bacteria, *P. protegens* PF5, was employed as a negative control which indeed resulted in low chlrorophyll levels accumulation in cotyledons during treatment similar to data obtained from sterile water, corroborating the results. Noteworthy, the M9 medium employed was recognized to stimulate microbial cytokinin synthesis *in vitro* and hence was employed to quantify *in vitro* expression of *miaA* from RZ2MS9 as a reference for *miaA* relative expression analysis.

Normalized Relative Quantities calculated using *miaA* expression data obtained from M9 broth treatment comparing to *miaA* expression data quantified in planta (leaves and roots tissues of bacterized maize seedlings cultivated in greenhouse) did not differ. This result is noteworthy because it showed *miaA* from RZ2MS9 is being expressed *in planta* at a statiscally similar rate as detected under *in vitro* conditions that favored cytokinin synthesis. Therefore, RZ2MS9 cytokinin production is suggested to be one of processes by which the bacterium promoted maize growth under evaluated conditions. It is relevant to observe that cytokinin production by *miaA* coded enzyme is aknowledged as part of bacterial machinery to cope with stress conditions,

such as a maize host with a defense system presumably activated according to up-expression of *lox1* and *pr1* in leaves also during RZ2MS9 maize colonization. Thus, it is not possible to know if putative cytokinin production by the bacterium is merely a part of microbial stress mitigating mechanism, being addressed to further studies. Nevertheless, it is plausible to infer that increased chlorophyll levels might increase plant photosynthetic capacity and host capacity to provide photoassymilates to endophytic bacteria, a process to be deeper investigated toward the benefit of benefic microbe use in agriculture.

4.5. CONCLUSIONS

This chapter progressed understanding of benefical relationship between tropical free-living Plant Growth Promoting Rhizobacterium (PGPR) *Bacillus sp.* and maize that resulted in growth promotion. Remarkably, *in vitro* maize leaves and roots gene expression profile reproduced greenhouse results for most of analyzed genes, validating proposed *in vitro* system for further studies. Up-expression of *lox, pr1*, and *β*-glucosidase *bglu60.1* in leaves of bacterized plants suggested activation of host defense mechanisms due to recognition of RZ2MS9.

RZ2MS9 modulated the expression of different genes in leaves and roots, comparing to control, favoring roots higher sink strength and growth at stage V2 under greenhouse conditions considering fresh matter, dry matter and soluble sugars increase and higher expression of auxin-responsive gene *iaa14,* sucrose synthase coding gene *susy* than control. Down-expression of ciszeatin-transglicosidase in bacterized roots suggested specific release of major maize cytokinin zeatin in the roots. Noteworthy, transcripts of cytokinin microbial biosynthetic gene *miaA* from RZ2MS9 were detected in similar levels in leaves and roots of bacterized maize seedlings cultivated in greenhouse and under cytokinin favoring culture conditions *in vitro*. Higher chlorophyll content was observed in RZ2MS9 bacterized maize seedlings cultivated in greenhouse, suggestting microbial interference into host hormonal balance through a mechanism to be further studied in which host release of hormone bound forms and production of cytokinin by the bacterium might participate. Maize genes studied with differential expression under RZ2MS9 interaction might contribute to further studies in other maize-PGPR systems.

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