UNIVERSIDADE D
COIMBRA

Inês de Sousa Rocha

SEED COATING WITH MICROBIAL INOCULANTS: A PATH TO SUSTAINABLE AGRICULTURE

Tese no âmbito do Doutoramento em Biociências, ramo de especialização em Biotecnologia, orientada pelo Doutor Rui Sérgio Viana Sodré de Oliveira, Doutor Miroslav Vosátka e Professora Doutora Helena Maria de Oliveira Freitas e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Agosto de 2019

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
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REVESTIMENTO DE SEMENTES COM INOCULANTES MICROBIANOS: UM CAMINHO PARA AGRICULTURA SUSTENTÁVEL

Inês de Sousa Rocha

Thesis in the scope of the Doctorate in Biosciences, area of specialization Biotechnology, supervised by Doctor Rui Sérgio Viana Sodré de Oliveira, Doctor Miroslav Vosátka and Professor Doctor Helena Maria de Oliveira Freitas, presented to the Department of Life Sciences of the Faculty of Sciences and Technology of the University of Coimbra.

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Table of Contents

LIST OF ABBREVIATIONS ..... **I**

LIST OF TABLE AND FIGURES ..... **IV**

ABSTRACT ..... **X**

RESUMO ..... **XIII**

CHAPTER 1 - GENERAL LITERATURE REVIEW **1**

INTRODUCTION 3

Sustainability 3

Soil-Plant-Microbes Interactions 4

Plant Growth Promoting Rhizobacteria 6

Arbuscular Mycorrhizal Fungi 8

Microbial Consortia 10

Microbial Inoculants and Inoculation Methods 10

BACKGROUND LITERATURE 13

Seed Coating with Beneficial Microbes 13

Ingredients, Types and Equipment 13

Formulation and Microbial Survival 15

Delivery of Beneficial Microbes 16

Comparison of Seed Coating with Other Methods 18

Agricultural Applications 19

RESEARCH FOCUS, OBJECTIVES AND THESIS OUTLINE 26

CHAPTER 2 - SEED COATING WITH INOCULA OF ARBUSCULAR MYCORRHIZAL FUNGI AND PLANT GROWTH PROMOTING RHIZOBACTERIA FOR NUTRITIONAL ENHANCEMENT OF MAIZE UNDER DIFFERENT FERTILIZATION REGIMES **30**

ABSTRACT 32

INTRODUCTION 32

MATERIALS AND METHODS 33

Soil and Plant Material 33

Inoculum Preparation and Seed Coating 34

Experimental Design 34

AM Fungal Analysis 35

PGPR Analysis 36
Plant Analysis 36
Statistical Analysis 37
RESULTS 37
Efficiency of Seed Coating as an Inoculum Delivery System 37
Growth and Nutritional Status of Maize Inoculated via Seed Coating 39
DISCUSSION 43
CONCLUSIONS 45

CHAPTER 3 - GROWTH AND NUTRITION OF COWPEA (*VIGNA UNGUICULATA*) UNDER WATER DEFICIT AS INFLUENCED BY MICROBIAL INOCULATION VIA SEED COATING 47

ABSTRACT 49
INTRODUCTION 49
MATERIALS AND METHODS 50
Seeds and Soil Material 50
Microbial Inocula and Seed Coating 50
Experimental Design 51
Gas Exchange Parameters 52
Chlorophylls and Carotenoids Content 52
Biomass Production, Seed Yield and Nutrients Acquisition 52
Mycorrhizal Development 53
Statistical Analysis 53
RESULTS 53
Plant Growth, Yield and Nutrients Concentration 53
Mycorrhizal Root Colonization 58
Leaf Parameters 58
DISCUSSION 63
CONCLUSIONS 65

CHAPTER 4 - SEED COATING WITH ARBUSCULAR MYCORRHIZAL FUNGI FOR IMPROVED FIELD PRODUCTION OF CHICKPEA 67

ABSTRACT 69
INTRODUCTION 69
MATERIALS AND METHODS 71
Arbuscular Mycorrhizal Fungal Inocula and Seed Coating 71
Experimental Design 71
Plant Measurements 72
Crude Protein and Fiber, Fat and Ash Grain Content Analyses 72
Mycorrhizal Development 72
Statistical Analysis 73
RESULTS 73

Growth Parameters 73
Grain Quality 75
Mycorrhizal Colonization 75
DISCUSSION 76
CONCLUSIONS 78

CHAPTER 5 – USING MICROBIAL SEED COATING FOR IMPROVED COWPEA PRODUCTIVITY UNDER LOW-INPUT AGRICULTURAL SYSTEM 80

ABSTRACT 82
INTRODUCTION 82
MATERIALS AND METHODS 83
Seeds, Microbial Inocula and Coating 83
Field Experimental Conditions and Design 84
Plant, Soil, and Grain Analyses 85
Mycorrhizal Root Colonization 85
Bacterial Detection in Cowpea Rhizosphere 85
Statistical Analysis 86
RESULTS 87
Cowpea Productivity and Grain Quality 87
Soil Physicochemical Properties 89
Microbial Colonization 89
DISCUSSION 90
CONCLUSIONS 92

CHAPTER 6 - GENERAL CONCLUSIONS AND FUTURE WORK 94

GENERAL CONCLUSIONS 96
Seed Coating as a Microbial Deliver System 96
Microbial Influence According to Fertilization and Water Deficit Regime 98
Microbial Seed Coating for Field Application 98
FUTURE WORK 99

REFERENCES 102

List of Abbreviations

ACC - 1-aminocyclopropane-1-carboxylate

AM - arbuscular mycorrhizal

ANOVA - analysis of variance

ASM - acibenzolar-S-methyl

BCA - biological control agents

CFU - colony-forming unit

Ci - intercellular CO_2 concentration

CoatPMR - cowpea coated seeds with *Pseudomonas libanensis* TR1+ multiple-isolates of *Rhizophagus irregularis*

CoatPR - cowpea coated seeds with *Pseudomonas libanensis* TR1 + *Rhizophagus irregularis* BEG140

D0 - no water deficit

D1 - moderate water deficit

D2 - severe water deficit

DAS - days after sowing

DNA - deoxyribonucleic acid

e.g. - (L. exempli gratia) for example

EN - European standard

et al. - (L. et alia) and other

F - fertilization

F0 - no fertilization

F1 - 80% strength Hoagland solution with 20% of phosphorus

F2 - full strength Hoagland solution

FAM - fungos arbusculares micorrízicos

FAO - Food and Agriculture Organization

FAOSTAT - Food and Agriculture Organization Statistics

FCT - Portuguese Foundation for Science and Technology

GDW - grain dry weight

gs - stomatal conductance

I - inoculation

IAA - indole acetic acid

ICP-OES - inductively coupled plasma optical emission spectrometry

INIAV - National Institute for Agrarian and Veterinary Research

IPCC - Intergovernmental Panel on Climate Change

ISO - International standard

LB - Luria Bertani

MIXcoat - cowpea coated seeds with *Rhizophagus irregularis* PH5 + *Pseudomonas putida* GP

MPCP - microrganismos promotores de crescimento de plantas

MPN - most probable number

MRcoat – chickpea coated seeds with multiple-isolates of *Rhizophagus irregularis*

NP - Portuguese norm

PBM - plant beneficial microbes

PCR - polymerase chain reaction

PFcoat - maize coated seeds with *Pseudomonas fluorescens* F113

PFsoil - maize conventionally inoculated with *Pseudomonas fluorescens* F113

PGPM - plant growth promoting microorganisms

PGPR - plant growth promoting rhizobacteria

Pn - steady-state net photosynthesis A

POCH - Programa Pperacional do Capital Humano

PPcoat - cowpea coated seeds with *Pseudomonas putida* GP

R+PFcoat – maize coated seeds with *Rhizophagus irregularis* BEG140 + *Pseudomonas fluorescens* F113

R+PFsoil – maize conventionally inoculated with *Rhizophagus irregularis* BEG140 + *Pseudomonas fluorescens* F113

Rcoat - chickpea coated seeds with *Rhizophagus irregularis* BEG140

Rcoat - maize coated seeds with *Rhizophagus irregularis* BEG140

RE - relative effectiveness

RH - relative humidity

RIcoat - cowpea coated seeds with *Rhizophagus irregularis* PH5

RLC - root length colonized

RPCP - rizobactérias promotoras de crescimento de plantas

Rsoil - maize conventionally inoculated with *Rhizophagus irregularis* BEG 140

SAR - systemic acquired resistance

SDW - shoot dry weight

SOM – soil organic matter

TM - thiamethoxam

Tr - transpiration rate

WR - water regime

WUE - water use efficiency

List of Table and Figures

Chapter 1 – General Literature Review

Tables

Table 1.1. Methods of application of microbial inoculants. 12

Figures

Figure 1.1. Sustainable agriculture comprises environmental, social, and economic concerns equally (created with Mind the Graph®).....4
Figure 1.2. Interactions between plant, soil and microorganisms (created with BioRender®). ...5
Figure 1.3. Bipartite network of interactions between plant beneficial microbes (PBM) and agricultural crops (from a total of 191 papers published between 1960 and 2019). Each colored line represents a specific association. In each case, the size of boxes is proportional to the number of interactions considered (a single study can include several interactions). Plant growth promoting rhizobacteria (PGPR) (blue), *Trichoderma* (green), rhizobia (red), arbuscular mycorrhizal (AM) fungi (yellow) and others [fungi (e.g. *Aspergillus* spp., *Beauvaria bassiana*) and the oomycete *Pythium oligandrum*] (purple). Percentages represent the proportion of interactions where the specific groups of PBM or plant species are participating. 18
Figure 1.4. Scale of experiments of seed coating inoculation, expressed as percentage of studies (from a total of 191 papers published between 1960 and 2019)..... 24
Figure 1.5. World map representing the number of studies dealing with seed coating with plant beneficial microbes by country and continent (from a total of 191 papers published between 1960 and 2019). Charts (green bars) indicate the number of published studies by continent, organized by intervals. Intervals correspond to decades (1990-99, 2000-10, and 2010-20), with the exception of the first interval, which collects studies of a larger period (1960-89) due to the low publication record during this period. Note the different scale of the y axis in the Asia chart. 25
Figure 1.6. Chapters and schematic outline of the PhD thesis 28

Chapter 2 – Seed coating with inocula of arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria for nutritional enhancement of maize under different fertilization regimes

Tables

Table 2.1. Experiment B - Percentage of root length colonized (% RLC), arbuscule (A %) and vesicle (V %) abundances of *Rhizophagus irregularis* (Rcoat) and *R. irregularis* and *Pseudomonas fluorescens* consortium (R+PFcoat) inoculated via seed coating in the roots of maize under no fertilization (F0), reduced fertilization (F1) and full fertilization (F2)..... 38

Table 2.2. Experiment B - Main effects of the factors inoculation and fertilization and two-way ANOVA *F*-values and significances for shoot and root biomass of maize. 40

Table 2.3. Experiment B - Shoot nutrient concentrations of *Zea mays* L. under different inoculation treatments via seed coating [Non-inoculated controls (C), *Rhizophagus irregularis* (Rcoat), *Pseudomonas fluorescens* (PFcoat) and a consortium of *R. irregularis* and *P. fluorescens* (R+PFcoat)] and fertilization regimes [no fertilization (F0), reduced fertilization (F1) and full fertilization (F2)]..... 41

Table 2.4. Experiment B - Main effects of the factors inoculation and fertilization and two-way ANOVA *F*-values and significances for shoot nutrient concentrations of *Zea mays* L..... 42

Figures

Figure 2.1. Experiment A - Percentage root length colonized (% RLC), arbuscule (A %) and vesicle (V %) abundances in the roots of maize conventionally inoculated in the soil with *Rhizophagus irregularis* (Rsoil) or *R. irregularis* + *Pseudomonas fluorescens* (R+PFsoil) or inoculated via seed coating (Rcoat and R+PFcoat). Values are means ± 1 SE. There were no significant differences according to Duncan's Multiple Range test at $P < 0.05$..... 38

Figure 2.2. Experiment B - Shoot and root dry weight of maize obtained from coated seeds non-inoculated (control), inoculated with *Rhizophagus irregularis* (Rcoat), *Pseudomonas fluorescens* (PFcoat) and a consortium of *R. irregularis* and *P. fluorescens* (R+PFcoat) under no fertilization (F0), reduced fertilization (F1) and full fertilization (F2). Values are means (±1 SE) followed by letters that indicate significant differences according to Duncan's Multiple Range test at $P < 0.05$..... 39

Chapter 3 – Growth and nutrition of cowpea (*Vigna unguiculata*) under water deficit as influenced by microbial inoculation via seed coating

Tables

Table 3.1. Biomass production and seed yield of *cowpea* under different inoculation treatments [non-inoculated (Control) *Rhizophagus irregularis* (RIcoat), *Pseudomonas putida* (PPcoat) and the mix *R. irregularis* + *P. putida* (MIXcoat)] and no water deficit (D0), moderate water deficit (D1) and severe water deficit (D2). 55

Table 3.2. Main effects of the factors inoculation and water regime and two-way ANOVA *F*-values and significances for biomass production, seed yield and nutrient shoot concentration of cowpea. 56
Table 3.3. Main effects of the factors inoculation and water regime and two-way ANOVA *F*-values and significances for leaf parameters and chlorophyll and carotenoids contents of cowpea. 62

Figures

Figure 3.1. Effects of different inoculation treatments [non-inoculated (Control), with *Rhizophagus irregularis* (RIcoat), *Pseudomonas putida* (PPcoat) and the mix *R. irregularis* + *P. putida* (MIXcoat)] and water regimes on N (A), P (B) and K (C) shoot concentration in cowpea. Values are means ± 1 SE and letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. 57
Figure 3.2. Percentage of root length colonization (% RLC) in the roots of cowpea inoculated with *Rhizophagus irregularis* (RIcoat) or the mix *R. irregularis* + *Pseudomonas Putida* (MIXcoat) via seed coating under different water regimes. Values are means ± 1 SE and letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. 58
Figure 3.3. Effects of microbial inoculation [non-inoculated (control), *Rhizophagus irregularis* (RIcoat), *Pseudomonas putida* (PPcoat) and the mix of *R. irregularis* + *P. putida* (MIXcoat)] and water regime on Pn (A), gs (B), Ci (C), Tr, (D) and water use efficiency (WUE) (E) of cowpea. Letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. 60
Figure 3.4. Chlorophyll a (a), chlorophyll b, (b) and carotenoids (c) leaf concentrations of cowpea under different inoculation treatments [non-inoculated control (control) and inoculated with *Rhizophagus irregularis* (RIcoat), *Pseudomonas putida* (PPcoat) and the mix of *R. irregularis* + *P. putida* (MIXcoat)] and water regimes. Letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. 61

Chapter 4 – Seed coating with arbuscular mycorrhizal fungi for improved field production of chickpea

Tables

Table 4.1. Growth and productivity parameters of chickpea in different inoculation treatments [control, *Rhizophagus irregularis* (Rcoat), a mixture of *R. irregularis* isolates (MRcoat) under greenhouse and field conditions. 74
Table 4.2. Harvest index (ratio of grain dry weight to shoot dry weight) of chickpea and relative effectiveness of inoculation (ratio of shoot dry weight of inoculated plant to shoot dry weight of non-inoculated plants) under field conditions. 74

Table 4.3. Chickpea grain content in different inoculation treatments [control, *Rhizophagus irregularis* (Rcoat), mixture of *R. irregularis* isolates (MRcoat) under greenhouse and field conditions. 75

Figures

Figure 4.1. Representation of productivity parameters of chickpea in different inoculation treatments [control, *Rhizophagus irregularis* (Rcoat), a mixture of *R. irregularis* isolates (MRcoat)], under greenhouse and field conditions. Radial graphs represent results relative to the higher value (indicated as 100%) for each productivity parameter. 75
Figure 4.2. Percentage of root length colonization (% RLC) of chickpea non-inoculated (control) and inoculated with *Rhizophagus irregularis* BEG140 (Rcoat) or mixture of *R. irregularis* isolates (MRcoat) via seed coating in greenhouse (A) and field (B) trials. Columns are means ± 1 SE and letters indicate significant according to Duncan's Multiple Range test at $P < 0.05$. 76

Chapter 5 – Using microbial seed coating for improved cowpea productivity under low-input agricultural system

Tables

Table 5.1. Growth and productivity parameters of cowpea in different treatments [control, *Pseudomonas libanensis* + *Rhizophagus irregularis* (coatPR), *P. libanensis* + mixture of *R. irregularis* (coatPMR) under field conditions..... 87
Table 5.2. Cowpea grain content in different inoculation treatments [control, *Pseudomonas libanensis* + *Rhizophagus irregularis* (coatPR), *P. libanensis* and a mixture of *R. irregularis* isolates (coatPMR)]..... 89
Table 5.3. Soil physicochemical properties after plant growth. 89

Figures

Figure 5.1. Harvest index (ratio of seeds to shoot dry weight) of cowpea in different inoculation treatments [control, *Pseudomonas libanensis* + *Rhizophagus irregularis* (coatPR), *P. libanensis* + mixture of *R. irregularis* (coatPMR)..... 88
Figure 5.2. Representation of harvesting productivity parameters of cowpea in different inoculation treatments [control, coated with *Pseudomonas libanensis* + *Rhizophagus irregularis* (coatPR) or coated with *P. libanensis* + a mixture of different *R. irregularis* isolates (coatPMR)]. Radial graphs represent results relative to the higher value (indicated as 100%) for each productivity parameter. 88
Figure 5.3. Mycorrhizal colonization, expressed as percentage of root length colonization (% RLC) in the roots of cowpea non-inoculated (control) and inoculated with *Pseudomonas libanensis* + *Rhizophagus irregularis* BEG140 (coatPR) or *Pseudomonas libanensis* + mixture of *R. irregularis* BEG141, BEG236, DAOM 197198, KW and AS (coatPMR) via seed coating. Columns are means

± 1 SE and letters indicate significant differences ($P < 0.05$) according to Duncan's Multiple Range test. 90

Abstract

Keywords: Arbuscular Mycorrhizal Fungi; Field trials; Plant Growth Promoting Rhizobacteria; Plant Beneficial Microbes; Seed Coating; Sustainable Agriculture

The interest in plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal (AM) fungi for agricultural purposes (e.g. enhancement of crop yield and nutrition, promotion of plants resilience to abiotic stress) is rising. Yet, large-scale applications of these microbes have been hampered by the lack of data on their field performance and the feasibility of the inoculation methods, especially in the case of AM fungi. Seed coating, a technique in which seeds are covered with minor amounts of exogenous materials (including microbial inoculants), is a potential tool to deliver microbes at large-scale. This technique has been gaining attention in the agricultural sector. A literature review revealed that seed coating has been applied to more than 50 plant species (e.g. wheat, tomato, maize, melon, bean, clover), including seeds with different characteristics (dimensions, forms, textures). Mostly studied for the application of various species of PGPR (especially from the genera *Pseudomonas* and *Bacillus*), seed coating is not so frequently explored for inoculation of AM fungi or microbial consortia. The improvement of crop productivity and protection of plants against pathogens have been the main focus of research on microbial seed coating, while a smaller portion has been aimed at enhancing crops resistance to abiotic stresses.

One of the main goals of this PhD thesis was to evaluate seed coating as a deliver system for the inocula of AM fungi and PGPR. Overall, seed coating allowed the application of minor amounts of AM fungi and PGPR to the seeds of three selected agricultural crops: maize, cowpea and chickpea. Further, by comparing inoculation of AM fungi through direct soil inoculation (conventional) with seed coating, similar AM root colonization was obtained despite a reduction in the amount of applied inocula. Contrary to AM fungi, the presence of PGPR coated on the seeds could not be confirmed in the rhizosphere and roots of the inoculated crops.

It is well known that both AM fungi and PGPR have the ability to improve soil fertility and enhance plant nutrition, which can bring benefits for plant growth and development. By increasing nutrient availability and nutrient use efficiency, these plant beneficial microbes (PBM) can assist farmers to reduce their dependence on chemical fertilizers. The results obtained in this thesis showed that coating seeds with PGPR and AM fungi had a significant impact on plant shoot nutrient concentrations under different fertilization regimes. For instance, maize seeds coated with AM fungi (*Rhizophagus irregularis)* increased shoot nutrient concentration (nitrogen, phosphorus, potassium, magnesium and zinc), comparing with non-inoculated plants. Nutrient contents on

maize shoot were boosted by *R. irregularis* inoculation, particularly in the treatments where fertilization was reduced or absent. On the contrary, maize coated with PGPR (*Pseudomonas fluorescens)* presented most of the nutritional increments when full fertilization was applied. Nevertheless, in both inoculation treatments, despite the nutrient enhancements, no improvement in plant biomass was obtained. These results confirm that PBM can increase plant nutrient uptake.

PGPR and AM fungi are known to confer drought resistance to plants. Coating seeds with PGPR (*Pseudomonas putida)* showed a general positive influence in the plant productivity, especially under moderated water deficit. Seed coating with AM fungi (*R. irregularis* singly or in consortia with *P. putida*) promoted nutrient uptake, leaf pigment contents and gas exchange parameters of cowpea, yet mostly when plants where under no water deficit. Mainly, these results emphasized the importance of selecting the PBM that better potentiate plant resilience to abiotic stresses, in order to obtain the best benefits from the inoculation.

Field experiments are essential to validate the benefits of microbial seed coating and its feasibility for large-scale applications. A comparison between chickpea coated with a single AM fungal isolate of *R. irregularis* and multiple isolates of the same fungal species under greenhouse and field conditions showed that plants inoculated with multiple AM fungal isolates performed better (e.g. higher biomass, increased grain yield) than those inoculated with a single AM isolate. Seed coating proved to be an appropriate tool to deliver AM fungi with benefits for chickpea plants at both experimental scales, but particularly relevant under field conditions. The mixture of multiple *R. irregularis* isolates was also used in consortium with *Pseudomonas libanensis* for coating cowpea seeds. This treatment significantly improved crop productivity in comparison with non-inoculated plants and plants inoculated with *R. irregularis* single-isolate + *P. libanensis*. The results showed improvements in grain lipid content, soil physicochemical properties (pH and soil organic matter), and crop yield under low-input agricultural systems. AM fungi and PGPR should be selected for microbial seed coating formulation according to their affinity with the host crop, growing conditions (e.g. soil properties) and farming practice (e.g. irrigation and fertilization), in order to obtain economical profits.

This thesis is a contribution to the knowledge on microbial seed coating and highlights the potential of seed coating as a microbial delivery tool and the benefits of its use in different agricultural conditions. Microbial seed coating can be of great use for sustainable agricultural systems. Yet, in order to allow its large-scale application as a cost-effective technique for PGPR and AM fungi inoculation, further development is necessary.

Resumo

Palavras-chave: Agricultura Sustentável; Experiência de campo; Fungos Arbusculares Microrrízicos; Microrganismos Benéficos para Plantas; Revestimento de Sementes; Rizobactérias Promotoras de Crescimento de Plantas;

O interesse em rizobactérias promotoras de crescimento de plantas (RPCP) e fungos arbusculares micorrízicos (FAM) para fins agrícolas (*e.g.* melhoramento do valor nutricional e rendimento de culturas, promoção de resiliência de plantas a factores abióticos) tem vindo a aumentar. Contudo, a aplicação em larga escala destes microrganismos tem sido dificultada pela escassez de dados sobre a performance destes em campo e viabilidade dos métodos de inoculação, em particular, no caso dos FAM. O revestimento de sementes, uma técnica que consiste em cobrir sementes com pequenas quantidades de materiais exógenos (incluindo inoculantes microbianos), representa uma potencial ferramenta para inocular microrganismos em grande escala. O revestimento de sementes tem vindo a ganhar importância no sector agrícola. Segundo a revisão da literatura realizada nesta tese, o revestimento de sementes foi usado em mais de 50 espécies de plantas (*e.g.* trigo, tomate, milho, melão, feijão, trevo), abrangendo sementes com diferentes características (dimensões, formas, texturas). Principalmente estudado para aplicação de várias espécies de RPCP (em particular do género *Pseudomonas* e *Bacillus*), o revestimento de sementes não tem sido tão frequentemente usado para inoculação dos FAM ou consórcios microbianos. O aumento da produtividade e proteção de culturas agrícolas contra agentes patogénicos têm sido o principal foco da investigação sobre revestimento de sementes com microrganismos benéficos. Por outro lado, os estudos referentes ao melhoramento da resistência de plantas a stresses abióticos através do revestimento de sementes têm sido consideravelmente menores.

Um dos principais objectivos desta tese foi avaliar a técnica de revestimento de sementes como método de inoculação para FAM e RPCP. De uma forma geral, o revestimento de sementes permitiu a aplicação de pequenas quantidades de FAM e RPCP em sementes de três culturas: milho, feijão-frade e grão-de-bico. Uma comparação entre inoculação directa no solo (convencional) e revestimento de sementes com FAM, mostrou que, apesar da redução na quantidade de inoculo aplicado, a eficiência do fungo na colonização nas raízes da planta alvo foi similar. Contrariamente aos FAM, a presença de RPCP na rizosfera e raízes das culturas selecionadas não foi confirmada.

É de conhecimento geral que tanto os FAM como RPCP têm a capacidade de melhorar a fertilidade do solo e o estado nutricional das plantas, o que pode ser de grande proveito para o

desenvolvimento e crescimento destas. Através do aumento da disponibilidade ou eficiência de utilização dos nutrientes, estes microrganismos promotores de crescimento de plantas (MPCP) podem ajudar os agricultores a reduzir a dependência de fertilizantes sintéticos. De acordo com os resultados obtidos nesta tese, sementes revestidas com RPCP e FAM tiveram um impacto significativo no estado nutricional das plantas quando submetidas a diferentes regimes de fertilização. Por exemplo, sementes de milho revestidas com FAM (*Rhizophagus irregularis*) apresentaram um aumento significativo na concentração de nutrientes na parte área (azoto, fósforo, potássio, magnésio e zinco), quando comparado com plantas não inoculadas. A concentração de nutrientes, na parte área do milho, foi estimulada pela inoculação de *R. irregularis*, em particular, em tratamentos com fertilização reduzida ou ausente. Pelo contrário, o aumento na concentração de nutrientes em milho revestido com RPCP (*Pseudomonas fluorescens*) foi superior quando plena fertilização foi aplicada. Contudo, apesar do incremento nutricional, em geral, não se verificaram aumentos a nível da biomassa da planta em ambas as inoculações. Estes resultados confirmam que os inoculantes podem influenciar de forma positiva a absorção de nutrientes pelas plantas.

RPCP e FAM são conhecidos por conferir resistência a plantas sobre stress hídrico. O revestimento de sementes com RPCP (*Pseudomonas putida*) mostrou, em geral, um efeito benéfico na productividade do feijão-frade, em especial, quando submetido a deficit hídrico moderado. Por sua vez, o revestimento de sementes com FAM (*R. irregularis*) individual ou em consórcio com *P. putida* promoveu a absorção de nutrientes pela planta, o conteúdo de pigmentos nas folhas e parâmetros de troca gasosa, contudo, na sua maioria na ausência de stress hidríco. Estes resultados realçam a importância de selecionar os MPCP que melhor fomentem a resiliência das plantas a stresses abióticos, a fim de tirar melhor partido da inoculação.

Experiências de campo são indispensáveis para corroborar os benefícios do revestimento de sementes com microrganismos e a viabilidade para aplicações em grande escala. Uma comparação entre grão-de-bico revestido com um único isolado de *R. irregularis* e com uma mistura de vários isolados de *R. irregularis*, em estufa e em campo, mostrou que as plantas revestidas com múltiplos isolados tiveram um melhor desempenho (*e.g.* incremento na biomassa e na produção de grão). O revestimento de sementes mostrou ser uma ferramenta adequada para a inoculação de FAM com vantagens para a produção de grão-de-bico em ambas as escalas experimentais, em particular, para as condições de campo. A mesma mistura de isolados de *R. irregularis* foi usada em consórcio com *Pseudomonas libanensis* para revestir sementes de feijão-frade. Este tratamento aumentou significativamente a produtividade da cultura em comparação com plantas não inoculadas e plantas inoculadas com único tipo de isolados de *R. irregularis* + *P. libanensis*. Os resultados revelaram melhoramentos no conteúdo lipídico das sementes, nas propriedades físico-químicas do solo (pH e matéria orgânica do solo) e no rendimento da cultura, num sistema agrícola de baixo *input*. FAM e RPCP devem ser selecionadas de acordo com a sua afinidade com a planta alvo, condições de cultivo (*e.g.* propriedades do solo) e práticas agrícolas (*e.g.* irrigação, fertilização), de forma a obter lucros.

Esta tese contribui para aumentar o conhecimento sobre revestimento de sementes com microrganismos, e realça o potencial da técnica como ferramenta de inoculação de FAM e RPCP e os seus benefícios em diferentes condições agrícolas. O revestimento de sementes com microrganismos pode ser de grande interesse para sistemas agrícolas sustentáveis. Contudo, de forma a permitir o uso em larga escala, como um método eficiente para a inoculação de RPCP e FAM, é necessário apostar no seu melhoramento e desenvolvimento.

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Rocha I. 2019. Seed coating with microbial inoculants: a path to sustainable agriculture. PhD Thesis, University of Coimbra, Coimbra, Portugal

Chapter 1 - General Literature Review

This chapter is based on a review article submitted to *Frontiers in Plant Sciences* (under review): Rocha I., Ma Y., Souza-Alonso P., Vosátka M., Freitas H. and Oliveira R. S. Seed coating: a tool to deliver beneficial microbes to agricultural crops.

Introduction

More than food and feed production or worldwide employment, agriculture is a sector of great importance, where decisions and actions should foresee consequences to the people, environment and Earth. With the Green Revolution high-input agronomic practices have started to be implemented at large scale (e.g. high-yielding crops varieties, intensive irrigation and chemical input). Farmers were able to maximize production and reduce food prices, and for many years, a rise in agriculture productivity was possible (FAO, 2018a). Yet, high-intensification of agroecosystems based on the excessive use of synthetic inputs (e.g. fertilizers and pesticides) and water, led to reduced ecosystem functioning, environmental degradation, biodiversity loss and therefore contributed to climate change (Robertson et al., 2014; Tubiello et al., 2015; Kanianska, 2016). Noteworthy, crop and animal production and forestry, mainly deforestation, are responsible for about a quarter of total global greenhouse gas emissions (IPCC, 2014a). Consequently, increases in temperature, drought and other climate change effects that can trigger pest and disease incidence have a major impact on agriculture and food prices, especially affecting the disadvantaged and more vulnerable populations (IPCC, 2018).

Till 2050, the world population is expected to grow to 9.8 billion (United Nations, 2017), which will obligate to an increase of 60% in food supply (Grafton et al., 2015). A great share of the Earth's land surface is already being used for food production (FAOSTAT, 2016) and satisfying food demand through the expansion of these areas is no longer feasible to increase productivity (Foley et al., 2011). Besides the growing population (plus consequent food demand) and land scarcity, the future agriculture will be critically challenged by a set of pressures related to soil degradation and depletion, water shortage and pollution, loss of natural resources, energy scarcity and climate change (Hanjra and Qureshi, 2010; Power, 2010; Foley et al., 2011).

The current route of agricultural production and productivity is unmaintainable, and past intensive agricultural practices are no longer an option for future outcomes. Without a significant change, environmental integrity, social and economic cohesion and health security will be seriously compromised. Thus, a more sustainable path is needed.

Sustainability

The Green Revolution boosted agricultural production and productivity and saved many from famine (Khush, 2001), yet, presently, a new revolution is necessary, one that includes adaption to climate change and sustainable use of energy, water, biodiversity and ecosystem services (Conway and Barbier, 2013).

Agricultural sustainability aims at satisfying the needs of the present without compromising that of future generations, giving equal importance to environmental health, economic gain and social fairness (Figure 1.1) (Lichtfouse et al., 2009). Efficiency in the use, protection and

conservation of natural ecosystems, improvement of opportunities and social well-being for people and enhancement of ecosystems stress-resilience can lead to a high-productive, economically viable and environmentally-friendly agricultural system (FAO, 2018a). Sustainable agriculture is the route to safer and high-quality food, environmental protection and preservation, and social and economic equality.

Long-term sustainability in an agroecosystem includes reducing or eliminating the dependence of non-renewable synthetic input such as fertilizers and pesticides, and efficient management and conservation of the natural resources (e.g. soil, water), while maintaining or increasing agricultural productivity (Reganold and Watcher, 2016).

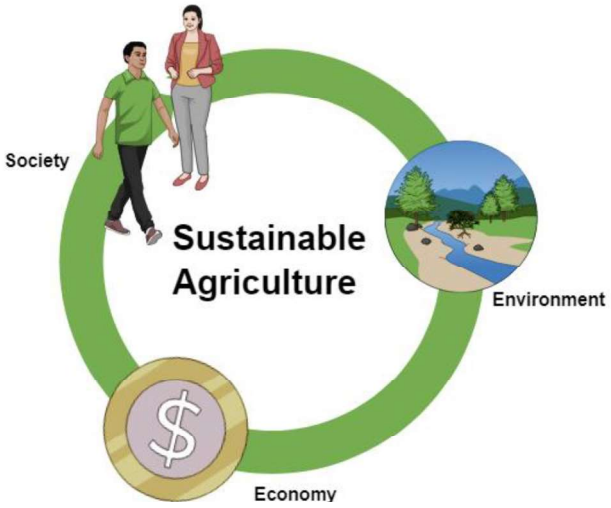

Figure 1.1. Sustainable agriculture comprises environmental, social, and economic concerns equally (created with Mind the Graph®).

Within the perspective of pursuing a more sustainable agricultural path, soil and its inhabitants may play a fundamental role (Busby et al., 2017; Glick, 2018). Soil organisms are responsible for several ecosystem functions and pivotal in the main elemental cycles (e.g. nutrients, carbon), yet their activities are still poorly explored in agricultural management strategies (Bender et al., 2016). Intensive agricultural practices, severely affect soil microbes. The abusive application of synthetic fertilizers is leading to simpler soil networks with less functional groups of soil biota (Tsiafouli, et al., 2015). Intensive soil tillage, repeated and intensive fertilization, application of pesticides, and low plant diversity, have been depauperating soil microbes in agroecosystems (Lupwayi et al., 2012; Prashar and Shah, 2016). Soil microbes can, however, be stimulated or directly used to favor agricultural purposes.

Soil-Plant-Microbes Interactions

Soils are complex, dynamic and biological-rich habitats (Bardgett and Van der Putten, 2014), where the major fraction of the living biomass is formed by microorganisms (Fierer, et

al., 2012). Soil microbes influence a large number of important processes, including carbon and nitrogen (N) cycling and soil formation (Van der Heijden et al., 2008). For instance, some steps in the N cycle such as fixation of atmospheric N_2, nitrification and denitrification are exclusively performed by microbes (Bender et al., 2016). These microorganisms interact with both soil and plants (Figure 1.2). Plants and microbes acquire nutrients from the soil and modify soil properties through organic matter deposition and metabolic activities (responsible for decomposition of organic matter, humus formation, etc.). Plants provide metabolites/nutrients to soil microorganisms and in its turn, soil microbes affect plant growth and performance by an array of direct and indirect mechanisms, e.g., enhancement of nutrients availability, production of enzymes and hormones, nutrient competition, protection against pathogens (Jacoby et al., 2017).

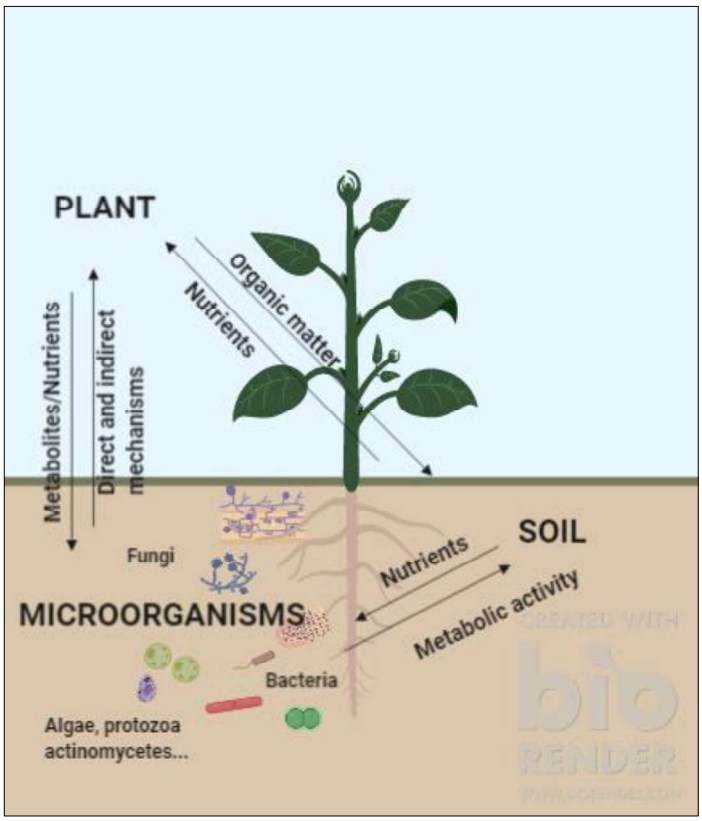

Figure 1.2. Interactions between plant, soil and microorganisms (created with BioRender®).

Plants offer diverse microhabitats where a wide range of microorganisms can form symbiotic relationships. Morphology, temperature, light or air exposure in different plants organs provide specific conditions that influence microbiota and its presence (Bulgarelli et al., 2013). A significant proportion of plant-microbe interactions occur in the space known as the rhizosphere. A hotspot of microbial activity, the rhizosphere is a playground and battlefield for soil borne pathogens and beneficial microbes (Raaijmakers et al., 2009). Plant-microbe interactions involve intricate trophic relationships conditioned by the "rhizosphere effect"

(Hartmann et al., 2008) that is mainly driven by rhizodeposition, from roots to the surrounding space. The release of organic compounds from plant roots dominates the communicative plant-microbe exchange in the rhizosphere (Bacilio-Jiménez et al., 2003). Colonization of the rhizosphere and root is the result of complex signal exchange between roots and microbes that will condition the net balance of the symbiotic relationship to be positive (mutualistic), neutral (commensalism) or negative (antagonistic). Consequently, the type of plant-microbe association will influence the development and fitness of the host plant (Yadav et al., 2015).

Soil microbes forming mutualistic interactions with plants (e.g. growth, nutritional value or resistance to various stresses) are of great interest for agricultural purposes (Berg, 2009; Singh et al., 2016; Zaidi and Khan, 2017). Many are the designations for these microorganisms, such as plant beneficial microbes (PBM), plant growth promoting microorganisms (PGPM), or more specifically, and according to the functions of their application, biofertilizers, biopesticides, stress bioalleviators, etc. Overall, these microbes can be classified according to their origin, location or function. Based on their origin, they can be grouped into prokaryotic [e.g. rhizobia, plant growth promoting rhizobacteria (PGPR) rhizobacteria, cyanobacteria] or eukaryotic [e.g. *Trichoderma*, arbuscular mycorrhizal (AM) fungi]. According to the location and degree of intimacy with plants, they are divided into free-living/rhizosphere microorganisms that depend on chemical compounds released into the rhizosphere, rhizoplane microbes that live in direct contact with plant tissues in the rhizoplane (root surface), and endophytic microbes that live inside plant tissues. Finally, microbes can have a direct or indirect role in nutrient and water uptake, synthesis of compounds, or protection of plants against biotic-abiotic stresses (Glick, 2014; de Souza et al., 2015; Pii et al., 2015; Berruti et al., 2016; Vejan et al., 2016). Currently, among PBM, there is a great interest in two main groups, PGPR and AM fungi. Nevertheless, other fungi such as *Trichoderma*, *Aspergillus* spp., *Beauvaria bassiana* and *Gliocladium virens* are commonly used as biofertilizers and biological control agents (BCA).

Plant Growth Promoting Rhizobacteria

Being one of the most common microbes present in soils, bacteria are not so evenly distributed, presenting higher concentrations in the rhizosphere, in comparison to bulk soil (Olanrewaju et al., 2017). About 2 to 5% of these rhizospheric bacteria promote plant growth (Antoun and Kloepper, 2001), and are, therefore, named PGPR (term created by Kloepper and Schroth in 1978). Depending on their proximity to the roots, PGPR can be rhizospheric, living near the root or in the root surface or endophytic, residing inside plant tissues (Vessey, 2003). Noteworthy, the definition of PGPR normally only contemplates free-living bacteria that are rhizosphere competent (even if they can invade roots and be endophytic), N-fixing bacteria such as rhizobia that are able to create specific symbioses with legume-plants forming specialized structures (root nodules), are normally not considered. Yet, because rhizobia can also colonize

the rhizosphere of non-host plants (non-legumes) and be endophytic, some authors regard them as PGPR (Siddiqui, 2006; Vargas et al., 2017).

PGPR can directly influence plant growth by facilitating nutrient uptake and/or regulating biosynthesis of phytohormones (Vejan et al., 2016). N, phosphorus (P) and iron (Fe) are essential nutrients, yet, not always available for uptake by plants (Glick, 2012). Mainly, two types of bacteria possess the ability to fixate atmospheric N and supply it to the plants, the legume-associated symbiotic bacteria that have plant-specificity and produce root nodules (e.g. *Rhizobium*) and the free-living N-fixers that do not possess specificity to plants (e.g. *Azospirillum* sp., *Azotobacter* sp., *Burkholderia* sp., *Bacillus* sp.) (Santi et al., 2013; Singh et al., 2017). P and Fe in soils normally exist in the insoluble form unavailable for plant uptake. PGPR allow P and Fe solubilisation in soils by releasing compounds (e.g. organic acids and enzymes) or producing siderophores (low-molecular-weight compounds that are capable of binding Fe), thereby increasing their availability for plant uptake (Vejan et al., 2016). Many agricultural soils are deficient in one or more of these nutrients, which led to the application and dependence on chemical fertilizers. Yet, the production of chemical fertilizers has environmental costs (e.g. pollution, use of non-renewable resources) (Glick, 2012; Santi et al., 2013). PGPR can be applied as biofertilizers due to their potential to improve nutrient use efficiency in low-nutrient agroecosystems or reduce application rates of chemical fertilizers (Shaharoona et al., 2008; Adesemoye and Kloepper, 2009; Duarah et al., 2011; Glick, 2012; Sessitsch and Mitter, 2015; Oliveira et al., 2016a). Further, PGPR can also function as phytostimulators by producing and regulating phytohormones (e.g. auxins, cytokinins, gibberellins, ethylene) that control processes such as seed germination, plant cell enlargement, division, flowering, and fruit set (Glick, 2014; Goswami et al., 2017). Phytohormones play a key role in plant growth and development as well as their response to environment conditions (Ahammed and Yu, 2016). In agriculture, phytohormones can be used not only with the purpose of growth stimulation but also to improve plant stress resistance (Fahad et al., 2015; Verma et al, 2016; Egamberdieva et al., 2017).

In a more indirect manner, PGPR can also promote plant development by acting as BCA or biopesticides against various plant pathogens. This includes several mechanisms of action such as synthesis of lytic enzymes (e.g. chitinases, cellulases, 1,3-glucanases, proteases), production of allelochemicals (e.g. antibiotics, hydrogen cyanide), competition for nutrients. Further, by inducing systemic resistance, a defense mechanism that confers protection against different pathogens, PGPR can improve plant resistance to biotic stress (Compant et al., 2005; Bakker et al., 2007; Glick, 2012). The use of these BCA in agriculture is of great interest since it can provide an opportunity to minimize the use of chemical pesticides and thus reduce negative environmental impacts and increasing food safety (Nadeem et al., 2014).

Not all PGPR are able to perform their beneficial effects on plants under abiotic stress conditions (e.g. drought, extreme temperatures, salinity, flooding, heavy metals), but some of them, not only tolerate stress, but have the ability to confer stress tolerance to host plants and

promote plant resilience in such stressful environments. These bacteria can act as stress bioalleviators to improve plant tolerance to abiotic stresses by various mechanisms such as lowering of stress-induced ethylene level, production of exopolysaccharides and hormones. Crop productivity is often challenged by abiotic stress such as drought and salinity that tend to be intensified by climate change (IPCC, 2014b). Thus the use of PGPR as bioalleviators can be of great relevance.

PGPR belong to diverse genera such as *Azospirillum*, *Azotobacter*, *Gluconacetobacter*, *Pseudomonas*, *Bacillus, Enterobacter*, *Klebsiella*, and *Serratia*. Among them, *Bacillus* and *Pseudomonas* are considered the predominant and the most broadly studied (Podile and Kishore, 2006). The diversity of PGPR strains in soils depends on different factors such as plant species, soil properties and nutrients availability (Verma et al., 2019). Moreover, the metabolic competences of bacteria that can influence plants differ among them (Bulgarelli et al., 2013; Vejan et al., 2016). Thus, PGPR application as microbial inoculants in agriculture comprises a selection of bacterial strains according to their function and abilities (e.g. facilitation of nutrients uptake, biological control of pathogenic agents, stress mitigation) and resistance to environmental conditions (e.g. plant species, soil).

Arbuscular Mycorrhizal Fungi

AM fungi belong to the phylum Glomeromycota and constitute a group of root obligate biotrophs that form symbioses with several plant species (including 80% of terrestrial plants) (Lekberg et al., 2013; Berruti et al., 2016). AM fungi are ancient soil microorganisms with different geographic distribution patterns, varying from local to global scale according to the fungal species (Rodriguez-Echeverría et al., 2017). Biotic (e.g. biological activity and biodiversity) and abiotic (e.g. soil physicochemical properties, temperature, humidity) factors can influence AM fungi richness in different ecosystems (Van Geel et al., 2018).

AM fungi develop different morphological structures such as hyphae, arbuscules, vesicles and spores (Smith and Read, 2008). Hyphae, filamentous formations that can be external or internal to plant roots, are responsible for nutrient acquisition, propagation and formation of arbuscules, vesicles and spores. In the soil, external hyphae can grow towards roots, thus establishing contact and developing along their surface. By producing swollen appressoria, hyphae penetrate the epidermal and cortical cells of plant roots, spreading along the cortex. Once entering inside root cortical cells, hyphae can branch and condense giving origin to branching structures, which are known as arbuscules. AM fungi are defined by the presence of these structures that increase the contact area between root and fungus and are considered the major site of resources exchange in the symbiotic relationship. Further, hyphae can also form inter- or intracellular lipid reservoirs (so-called vesicles) that can function as propagules besides the storage role (Souza, 2015; Varma et al., 2017). In general, AM fungi can propagate via hyphae or spores (Bever et al., 2001). AM spores are asexual spherical structures that develop thick walls

(multilayer) originated from hyphae (external or internal) or vesicles. They can function as storage structures, resting stages and propagules, and their wall organization can be useful to identify the fungus (Souza, 2015).

Plants colonized by AM fungi have the ability to explore larger soil volume through the ramification of fungal hyphae. This hyphal extension (mycelium) creates communicative bridges that increase root surface, thereby increasing nutrient acquisition, ameliorating soil physical properties (e.g. soil aggregation, soil moisture retention capacity) and offering a primary protective barrier against pathogens (Ryan and Graham 2002; Jeffries et al., 2003; Rillig and Mummey, 2006; Sikes, 2010; Bücking and Kafle, 2015). AM fungi can also influence plant roots architecture (e.g. increasing volume, depth and weight), and thus influencing root-soil interactions (Berta et al., 2002). These fungi are capable of solubilizing inorganic forms of nutrients (particularly P) by releasing organic acids or enzymes (Chen et al., 2007). According to Marschner and Dell (1994), about 80% of P taken up by AM plants is supplied by the fungal partner. In addition to their significant role in P acquisition, AM fungi can also increase the uptake of other macro- and micro-nutrients such as N, potassium (K), magnesium (Mg), copper (Cu) and zinc (Zn) (Clark and Zeto, 2000; Miransari, 2011; Garcia and Zimmermann, 2014; Chen et al., 2017; Zhang et al., 2017; Ingraffia et al., 2019).

Besides promoting plant growth by providing nutritional and structural benefits, AM fungi can also help plants cope with environmental stresses (e.g. drought, salinity, heavy metals) through different mechanisms such as regulation of plant nutrition (e.g. improvement of plant nutritional status, alteration of sodium and K uptake) and metabolites (e.g. proline, sugars, enzymes), adjustment of physiological processes (e.g. increase of stomatal conductance, transpiration and photosynthetic rates, osmotic adjustment) and improvement of water use efficiency (Porcel and Ruiz-Lozano, 2004; Hajiboland et al., 2010; Evelin et al., 2012; Miransari, 2011; Ruiz-Lozano et al., 2012; Seguel et al., 2013; Wu et al., 2013; Augé et al., 2015; Shamshiri and Fattahi, 2016). These mechanisms vary depending on AM-plant association as well as stress conditions (type and intensity) (Nadeem et al., 2014; Chun and Chandrasekaran, 2018).

The most studied AM fungal species are *Rhizophagus irregularis* (formerly *Glomus intraradices*), *Funneliformis mosseae* (formerly *Glomus mosseae*) and *Gigaspora* spp. (*G. rosea*, *G. margarita* and *G. gigantea*) (Malbreil et al., 2014). These fungi have been used in agriculture to promote crop growth under stress and non-stress conditions (Gamalero et al., 2008; Pellegrino and Bedini, 2014; Oliveira et al., 2017b; Pawar et al., 2018; Chun and Chandrasekaran, 2018; Rocha et al., 2019).

Like PGPR, AM fungi have a great potential to help reduce the dependence on agrochemicals and to assist crops against biotic and abiotic stresses. Yet, their inoculation efficacy may greatly depend on many factors, including compatibility with the target environment, competition with other soil microbes, time of inoculation and plant-fungus specificity (Pellegrino and Bedini, 2014; Berruti et al., 2016). Not only AM fungal proliferation and sporulation are strongly dependent

on host plants, but also they present different levels of host specificity (Klironomos, 2000; 2003; Johnson et al., 2003).

Microbial Consortia

Interactions between different beneficial microbes and host plants can be fundamental to maintain soil fertility and plant health, particularly in low input agriculture that relies on biological process rather than agrochemicals (Sessitsch and Mitter, 2015). Combinations of different PBM, as microbial consortia, can result in improved plant performance. PGPR have been shown to positively influence legume-rhizobia and plant-fungi interactions (Vessey, 2003; Mohamed et al., 2014; Korir et al., 2017). The combined use of PGPR and N-fixing bacteria can improve root growth, plant resilience to environmental stresses, and reduce N losses (Dal Cortivo et al., 2017). It is well known that PGPR can be used to ameliorate nodule formation in legumes when co-inoculated with rhizobia (Tilak et al., 2006) and enhance plant growth indirectly by optimizing the relationship between host plants and AM fungi. Ratti et al. (2001) found that *Bacillus polymyxa* and *Azospirillum brasilense* enhanced root colonization by *Glomus aggregatum* and improved biomass and P content of palmarosa grass when supplied with insoluble inorganic phosphate. Moreover, AM fungi can also associate with legumes where rhizobia are present to increase grain yield and protein content (Oliveira et al., 2017a; 2017b). For example, a consortium of *G. mosseae* and *Trichoderma harzianum* increased the yield and seed quality of different agricultural crops (Egberongbe et al., 2010; Nzanza et al., 2012). Notwithstanding, the application of microbial consortia does not necessarily entails positive interactions. Competition for nutrient and niche and production of antagonistic secondary metabolites can occur. Therefore, the selection of appropriate PBM to be applied in consortia is crucial.

Microbial Inoculants and Inoculation Methods

Application of microbial formulations (i.e. use of microbial inoculants such as PGPR and AM fungi) have been considered a possible strategy to enhance agricultural crop productivity and quality, reduce the excessive dependence on agrochemicals and increase resource (e.g. water, soil) use efficiency (Singh et al., 2016). The use of PBM in agriculture is not new; yet, greater interest has occurred in recent years due to the need for more sustainable crop production and human and environmental health protection (Ahmad et al., 2011; Timmusk et al., 2017). For instance, *Rhizobium*-based inoculants (legume-nodulation commercial formulations) have been used in agriculture for more than 100 years (Bashan, 1998; Deaker et al., 2004). The broad-scale of rhizobial inoculation began in the early 20th century and only more recently, strains of other PGPR such as *Bacillus*, *Pseudomonas*, and AM fungi started to be commercialized (Backer et al., 2018). One of the most important factors that support successful establishment and performance of microbial inoculants is inoculation (Bashan et al., 2014), and

thus, suitable and effective methods for large-scale delivery of inoculants are essential (Khan et al., 2010; Glick, 2012).

PBM are usually added to the soil (direct soil application), the seed (seed-applied inoculant) or the plant (e.g. foliar spray and root dipping) (Adholeya et al., 2005; Mahmood et al., 2016). Each inoculation method has advantages and disadvantages, depending on the amount of inoculants, availability of equipment, type of seed (e.g. size, shape and fragility), the presence of inhibiting compounds in the seed (e.g. fungicides, micronutrients and PBM) and cost (Deaker et al., 2004; Bashan et al., 2014). A summary of some of the most common techniques used in the different inoculation methods and their advantages and disadvantages is presented in Table 1.1.

In general, direct soil inoculation is used to introduce a large amount of microbial inoculant into the soil, avoiding damage of fragile seeds or protecting the inoculant from inhibiting compounds applied or produced by the seed (e.g. fungicides and antimicrobial compounds). It can be done either using solid, liquid or encapsulated formulations at the time of seeding (Malusà et al., 2012; Bashan et al., 2014). However, direct soil inoculation is not economically feasible in large-scale applications due to the high amount of microbial inoculum required (Deaker et al., 2004; Adholeya et al., 2005; Vosátka et al., 2012).

Inoculation of plants through root dipping or foliar application are techniques that demand large amounts of inoculant and, in the case of root dipping, plant nursery preparation is also required. On the other hand, seed inoculation can be a cost-effective way to deliver microbes in large-scale field applications (John et al., 2010; O'Callaghan, 2016). Seed inoculation delivers PBM to the rhizosphere of the target crop, where an intimate plant-microbe contact is established since germination (Philippot et al., 2013). Besides being a precise delivery system, seed inoculation can also be used to modify seed characteristics (e.g. shape, size and weight, etc.), making it easy to handle and sow (Halmer, 2008). Seed coating, a technique of seed inoculation, has the potential to be a cost-effective way to deliver microbes, requiring less inoculum than other inoculation methods.

Table 1.1. Methods of application of microbial inoculants.

Method	Technique	Advantages	Disadvantages	References
Direct soil inoculation	Granular/powder; Liquid inoculation; Immobilized microbial cells	Avoids damaging fragile seeds and cotyledons; Overcomes the adverse effect of pesticides and fungicides applied to seed; Small seeds can receive higher dose of inoculant	Requires specialized equipment for application and larger quantities of inoculants; Requires more storage area and transport; Expensive method	Van Elsas and Heijnen, 1990; Smith, 1992; Deaker et al., 2004; Adholeya et al., 2005; Bashan et al., 2014
Plant inoculation	Foliar spray; Root dipping	Direct application; Application of microbial inoculant with high concentration	Expensive; Requires large amount of inoculant; Laborious and time consuming	Adholeya et al., 2005; Mahmood et al., 2016
Seed inoculation	Seed soaking; Seed coating (Seed dressing; Film coating; Pelleting/encrusting; Slurry coating); Bio-priming	Practical and ready-to-use product; Fast, cheap and accurate; Require low amount of inoculant; Confers other beneficial characteristics to the seed	Poor survival of the inoculant (reduced shelf-life); Insufficient amount of microbial inoculant for small seeds (except for pelleting); Incompatibility of seeds treatments (e.g. fungicides); Seed coat lifted out of the soil during germination	Kaufman, 1991; Smith, 1992; Adholeya et al., 2005; Ehsanfar and Modarres-Sanavy, 2005; Deaker et al., 2012; Bashan et al., 2014; Mahmood et al., 2016

Background Literature

Seed Coating with Beneficial Microbes

Seed coating is the application of exogenous materials onto the surface of seeds with the aim of improving seed appearance and handling characteristics (e.g. seed weight and size) and/or delivering active compounds (e.g. plant growth regulators, micronutrients, and microbial inoculants) that can protect the seed against phytopathogens, increase germination and plant growth (Halmer, 2008; Pedrini et al., 2017). Inspired in the pharmaceutical industry, seed coating was first applied to cereal seeds in the 1930s and thereafter its large-scale commercial use began in the 1960s (Kaufman, 1991). Nowadays, seed coating is used by horticultural and crop industries worldwide and has earned its place in the global market (Pedrini et al., 2017). It is used for applying colors and tracers (e.g. fluorescent dyes), protectants (e.g. pesticides), soil adjuvants (e.g. soil hydrophilic materials and hydro absorbers), compounds that stimulate germination, growth and stress resistance (e.g. salicylic acid, gibberellic acid, and abscisic acid), macro and micronutrients and PBM inoculants (Scott, 1989; Ehsanfar and Modarres-Sanavy, 2005; Pedrini et al., 2017). Coating crop seeds with PBM allows a precise application of minor amounts of inocula at the seed-soil interface (Scott, 1989), ensuring that the PBM are readily accessible at germination and early development plant stages, stimulating healthy and rapid establishment, and consequently maximizing crop production (Colla et al., 2015a).

Ingredients, Types and Equipment

Seed coating can vary from simple on-farm applications to sophisticated and industrialized procedures. Although the processes used by farmers and industrial companies may differ, the principle is basically the same. Overall, it includes, seeds inside a container (e.g. rotating drum, cement-mixer), where a binder (e.g. adhesive compound), a filler (bulking agent) if needed and active ingredients (e.g. nutrients, protectants and PBM) are mixed (Scott, 1989; Accinelli et al., 2016; Padhi and Pattanayak, 2018). Fillers can be single or mixed components and the most commonly applied are peat (Georgakopoulos et al., 2002; Hartley et al., 2004; Hameeda et al., 2010), talc (Mukherjee and Sen, 1998; Sabaratnam and Traquair, 2002; Berninger et al., 2016) and lime (Brockwell and Phillips, 1970; Gault and Brockwell, 1980; Padhi and Pattanayak, 2018). These components can function as microbial carriers and modify seed size, shape and weight. Some ingredients like alginate can be used both as filler and binder (Heo et al., 2008; Khan et al., 2011; Anis et al., 2012; Lally et al., 2017). Recently, biochar and chitosan have been also considered as fillers/carriers for microbial seed coating (Głodowska et al., 2016; Głodowska et al., 2017; Ruiz-de-la-Cruz et al., 2017). Binders, natural or synthetic polymers such as methyl cellulose (Hartley et al., 2004; Haikal, 2008; Swaminathan et al., 2016; Amutha, 2017; Lopisso et

al., 2017), carboxymethyl cellulose (Sharma et al., 2003; Roesti et al., 2006; Nawar, 2007; Zhou et al., 2017), gum arabic (Kyei-Boahen et al., 2001; Ehteshamul-Haque et al., 2007; Dawar et al., 2008; Singh et al., 2014) or polysaccharide Pelgel (Jensen et al., 2000; Li et al., 2002; Ugoji et al., 2006) are generally added during or towards the end of the coating process in order to bind the exogenous materials and reduce the amount of dust in the final product (Pedrini et al., 2017). Some adhesives (e.g. gum arabic and xanthan gum) can also be used to extend the survival of PBM applied to seeds (Jambhulkar et al., 2016). The selection of the proper type and concentration of binder and filler is crucial for seed germination, plant development and viability of the applied microbial inoculant. Other characteristics such as availability, cost, origin and environmental impacts should also be taken into consideration when choosing the most adequate coating materials.

The classification of seed coating types is usually based on the weight, size, and grouping properties of the coated seeds. Most studies do not specify the type of coating used, yet when reported the most frequent are seed dressing, film coating and pelleting (Hartley et al., 2004; Shaharoona et al., 2008; Domaradzki et al., 2012; Accinelli et al., 2016; Cely et al., 2016; Jacob et al., 2016; Shahzad et al., 2017; Accinelli et al. 2018b; Rocha et al., 2019). Moreover, other terms such as slurry coating can also be found in the literature related to microbial seed coating (Pill et al., 2009; Hartley et al., 2013; Rozier et al., 2017, Rehman et al., 2018).

The most basic coating treatment is seed dressing, which refers to the application of finely milled solids dusted onto the surface of seeds in small amounts and it is normally used for pesticides application (Scott, 1989). Yet, some studies use the term seed dressing, not as a type but as synonym for seed coating (Shaharoona et al., 2008; Choi et al., 2016; Murphy et al., 2017; Shahzad et al., 2017). Film coating is considered as a more recent method and it consists on the application of a thin layer of external material with little change of the seed shape, size and weight (Halmer, 2000; 2008). It can be considered an improved version of slurry coating, where a solution or suspension is also applied onto the seeds, but in a less firm and uniform layer (Taylor et al., 2001; O'Callaghan, 2016). Also, film coating allows better treatment precision and minimizes the production of dust. It is considered a well-established technique for coating of several high-value horticultural species and other important agricultural crops, such as maize, sunflower, soybean and canola (Accinelli et al., 2016). In comparison with other seed coating types, film coating has a lower interference with seed germination and a prompter release of active components (Halmer, 2008). Finally, pelleting comprises fillers and liquid binders applied to the seed that may cause a significant increase in weight and volume. Pelleting usually modifies seed morphology into a spherical or ovoid shape, making it impossible to discriminate the initial seed shape (Halmer, 2000). If the original seed shape is still maintained the term used is encrusting (Pedrini et al., 2017). Pelleting and encrusting increase the amount of applied active ingredients and improve seed handling and sowing, especially for irregularly shaped seeds (Halmer, 2008).

Depending on the type of coating, specific equipment is considered. The rotating pan is the most common device used for seed coating (e.g. pelleting, encrusting, dressing and film coating) (Hartley et al., 2013; Oliveira et al., 2016b; Rouphael et al., 2017; Accinelli et al., 2018b; Rocha et al., 2019; in press). It usually consists in an inclined round pan rotating in slow motion, where materials are gradually added, followed by size sorting (sieving and screening) and then drying (Halmer, 2000; Pedrini et al., 2017). Film coating and encrusting can also be carried out using a fluidized or spouted bed, a cylindrical apparatus where seeds are kept in suspension by a constant vertical/bottom-up hot airflow, while being sprayed with coating materials. The warm airflow allows moisture evaporation. This is a slow and costly process (Robani, 1994). Another device used for most seed coating types is the rotary coater or rotor-stator, a cylindrical drum with two rotating base disks, a concave one, whose rotation causes seeds to move steadily along the drum walls; and a smaller one that allows the atomization and projection of liquid/slurry coating to the rotating seed mass (Pedrini et al., 2017). Unfortunately, the majority of scientific publications disclose scarce information regarding equipment and methodological details, with a considerable number reporting seed coating procedures performed by specialized companies (Ugoji et al., 2006; Diniz et al., 2009; Junges et al., 2013; Rozier et al., 2017).

Formulation and Microbial Survival

The formulation of microbial inoculants generally consists in 3 basic elements: the selected microorganism, a suitable carrier (that can be solid or liquid) and different additives. It is worth to note that factors such as incorrect inoculant formulation or limited shelf-life (i.e. inoculant viability on the seed surface) can hamper a wider use of seed coating (O'Callaghan, 2016). Formulation has a major impact on the microbial survival during the process of product elaboration, storage and application, in its efficiency once applied on the target plant and in the economic feasibility of the application (John et al., 2011; Herrmann and Lesueur, 2013). Although the formulation of microbial inoculants is a critical issue, little research has been conducted on this topic (Parnell, 2016). Georgakopoulos et al. (2002) evaluated pre-selected bacterial and fungal antagonists responsible for biological control of damping-off in sugar beet and cucumber with the intention of developing potential commercial formulations based on a peat carrier material for seed coating. *Pseudomonas* antagonists were the most effective biocontrol agents and survived for 2 years at ambient temperature in the peat formulation. Moreover, a biochar-based seed coating with *Bradyrhizobium japonicum* inoculum allowed the maintenance of a high bacterial population for over four months, which ensured efficient nodulation of soybean (Głodowska et al., 2017). Therefore, bacterial survival was strongly affected by the physical and chemical properties of biochar. In fact, out of five applied biochar carriers, only two provided suitable conditions to maintain bacterial viability for long periods of time (nine months). On the other hand, alginate beads can also be used as carriers, which allow a slow and constant release of bacteria. Bashan (1986) developed synthetic beads made of sodium alginate and skim milk,

which are biodegradable and have no negative impact on the environment. The final product that consists of lyophilized beads containing immobilized bacterial inoculants can be coated onto crop seeds and then stored at ambient temperature at least for 3 months without loss of bacterial viability. Under high humidity conditions and without any drying procedure, coated seeds with the immobilized bacteria maintained high viability, however, the downside was that seeds germinated before sowing. Maintaining the viability of PBM coated onto seeds can be challenging but it is essential for commercial applications. Nevertheless, the shelf-life of seeds coated with microbial inoculants, including the viability of both seeds and coated microbes, is still an overlooked topic in the literature.

Delivery of Beneficial Microbes

An analysis of the published literature since 1960 has showed that the great majority of studies on microbial seed coating were conducted with PGPR (Figure 1.3). Rhizobia and *Trichoderma* are also among the most studied microbial inoculants. Within PGPR, *Pseudomonas* and *Bacillus* are the most commonly applied genera, which are mainly used as plant growth promotors (Bashan, 1986; Junges et al., 2013; Kumar et al., 2015; Choi et al., 2016; Głodowska et al., 2016; Rehman et al., 2018) and BCA (Georgakopoulos et al., 2002; Pereira et al., 2007; Sim et al., 2008; Singh et al., 2012; Moussa et al., 2013; Zhou et al., 2018). Co-coating of *Pseudomonas* and *Bacillus* increased seed vigor and decreased the infection level of *Xanthomonas oryzae* pv. *oryzae* in rice (Palupi et al., 2017) and enhanced canola height and biomass under greenhouse and field conditions (Lally et al., 2017). As the most frequently used rhizobial genus, *Rhizobium* has also been successfully coated singly and in consortia with other PBM, which resulted in positive effects on plant growth and yield (Fatima et al., 2006; Dawar et al., 2008; Dal Cortivo et al., 2017; Zhou et al., 2017; Padhi and Pattanayak, 2018). In some cases, the application of a certain ingredient for seed coating can limit the positive role of *Rhizobium* in plants. Adams and Lowther (1970) assessed the combined effect of lime and *Rhizobium* spp. via direct soil inoculation and seed coating on the establishment and growth of different clover species. Direct soil inoculation significantly increased nodulation and caused a threefold rise in plant yield after 32 weeks. Lime also greatly improved nodulation and yield with less intensity compared to direct soil inoculation. Yet, coating of inoculated seeds with lime had little or no effect on clover nodulation or yield. In fact, inoculated seeds coated with lime seemed to display reduced rhizobial survival. Similarly, the application of certain fungicides [e.g. N-(tri-chloromethylthio)-4-cyclohexene-1,2-dicarboximide, Metalaxyl-M, Carbathiin, Oxycarboxin, and Thiram] to seeds can be harmful to *Rhizobium* spp. depending on the species or strain, bacteria-fungicide contact period prior to planting, fungicide concentration, and environmental variables (e.g. high temperatures and dehydration). The survival of *Rhizobium ciceri* that was coated onto chickpea seeds and simultaneously treated separately with 4 commercial fungicides under laboratory conditions was reduced, according with the applied fungicide. In pot experiments, the negative

effects of fungicides on *Rhizobium* sp. were less intense, due to the buffer effect of the rhizosphere soil or the possible migration of inoculated strains from the fungicide zones. Kyei-Boahen et al. (2001) described discrepancies between the obtained results and previous reports and highlighted the importance of selecting an adequate fungicide compatible with the specific *Rhizobium* strain for seed coating application. Despite its ability to increase plant productivity and nutrition under greenhouse experiments (Oliveira et al., 2016b; Rocha et al., 2019) and yield of different agricultural crops under field conditions (Cely et al., 2016), the potential of AM fungi inoculation via seed coating to enhance plant performance is still poorly explored (Figure 1.3). On the other hand, as the most used group of fungi for seed coating, *Trichoderma* shows great ability to increase seed germination and plant growth (Nawar, 2007; Domaradzki et al., 2012; Accinelli et al., 2016), and control pathogenic agents such as *Rhizoctonia solani* (Mihuta-Grimm and Rowe, 1986; Dawar et al., 2008; Haikal, 2008), *Pythium* spp. (Hadar et al., 1984; Sivan et al., 1984; Lifshitz et al., 1986; Taylor et al., 1991), *Sclerotium cepivorum* (McLean et al., 2005) and *Fusarium* spp. (Sivan and Chet, 1986; Sivan et al., 1987; Babychan and Simon, 2017) under greenhouse and field conditions. For instance, simultaneous seed coating with inocula of *G. intraradices*, *G. mosseae* and *Trichoderma atroviride* enhanced growth, nutrient uptake, grain yield and quality of winter wheat (Colla et al., 2015a). Other fungi such as *Aspergillus* spp., *G. virens* were inoculated via seed coating mainly for biocontrol purpose (Dawar et al., 2008; Haikal, 2008; Singh et al., 2012). Combining different PBM in consortia can improve plant growth and performance (Nadeem et al., 2014). However, only 19% of studies (from a total of 191 papers published between 1960 and 2019) used seed coating with more than one type of PBM. Singh et al. (2014) developed chickpea seed coating with different combinations of *Pseudomonas aeruginosa* PHU094, *T. harzianum* THU0816 and *Mesorhizobium* sp. RL091 using gum arabic as a binder. The aim was to evaluate the effectiveness and potential of the PBM to promote plant growth and phenolic acid biosynthesis in chickpea infected with the fungal pathogen *Sclerotium rolfsii*. The consortium led to superior plant growth and higher amounts of phenolic compounds in chickpea grown under biotic stress when compared to their single inoculations and untreated control. Equally, significantly reduced wilt incidence caused by *Ralstonia solanacearum* and higher fruit yield were observed when talc-based consortium formulation of *Trichoderma parareesei* + *Pseudomonas fluorescens* + *Bacillus subtilis* + *Azotobacter chroococcum* was applied onto tomato seeds (Nath et al., 2016). Besides, the co-inoculation can also have a negative impact on plant performance. According to Diniz et al. (2006), co-inoculation of *Trichoderma* spp., *B. bassiana*, *Metarhizium anisopliae* and AM fungi greatly reduced the germination of lettuce seeds. Sometimes single inoculation can perform better than co-inoculation with several microbes. For instance, Ma et al. (2019) reported no benefit of *R. irregularis* applied via seed coating in combination with soil inoculated *Pseudomonas libanensis* on cowpea performance. On the contrary, when singly inoculated, *P. libanensis* was effective in enhancing cowpea biomass and seed yield. So far it is not clear whether microbial consortia applied via seed coating can be advantageous. The most

appropriate microbial combinations according to the plant species and growing conditions should be selected and factors that affect the functioning of microbial consortia and their survival onto coated seeds must be investigated.

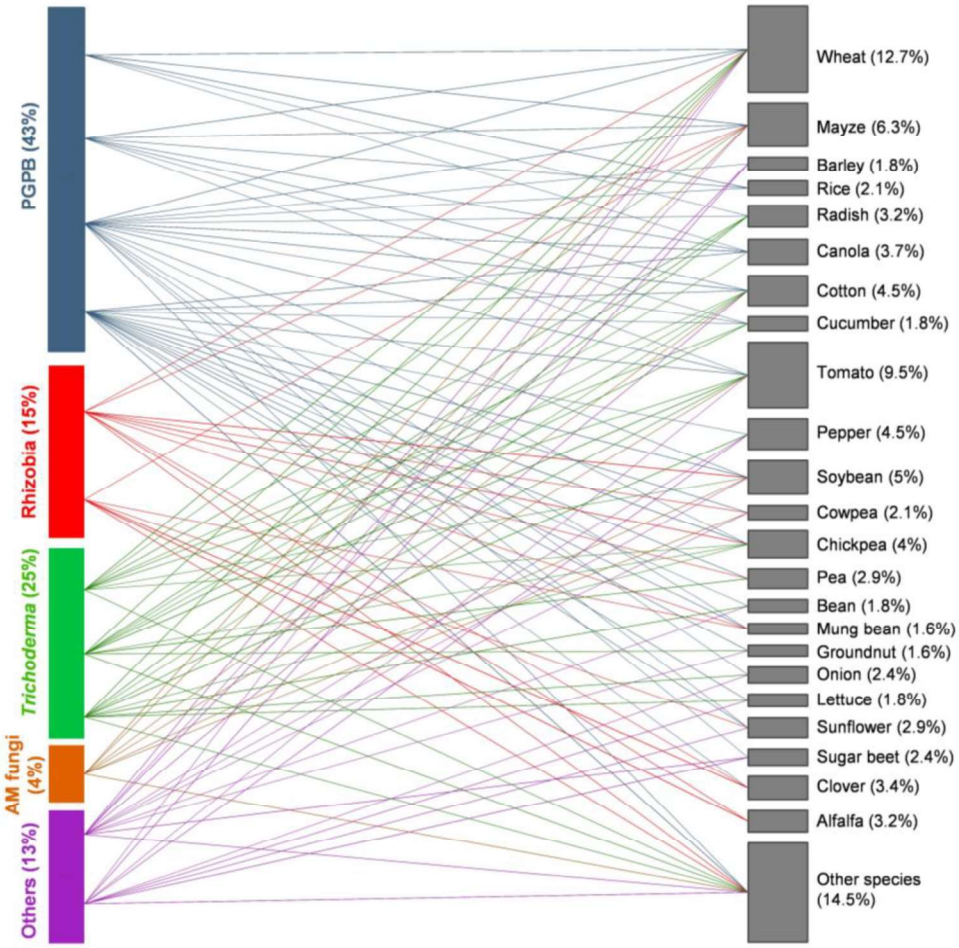

Figure 1.3. Bipartite network of interactions between plant beneficial microbes (PBM) and agricultural crops (from a total of 191 papers published between 1960 and 2019). Each colored line represents a specific association. In each case, the size of boxes is proportional to the number of interactions considered (a single study can include several interactions). Plant growth promoting rhizobacteria (PGPR) (blue), *Trichoderma* (green), rhizobia (red), arbuscular mycorrhizal (AM) fungi (yellow) and others [fungi (e.g. *Aspergillus* spp., *Beauvaria bassiana*) and the oomycete *Pythium oligandrum*] (purple). Percentages represent the proportion of interactions where the specific groups of PBM or plant species are participating.

Comparison of Seed Coating with Other Methods

Published data of comparisons between the efficiency and feasibility of inoculation of PBM via seed coating and other methods is still scarce. In a greenhouse experiment, after comparing seed coating of *Rhizobium* strains with soil drench application for the management of root-knot nematode *Meloidogyne incognita* on soybean, Ahmed et al. (2016a) found that seed dressing was more effective in controlling the reproduction of *M. incognita* and increasing plant height, fresh

and dry root and shoot weight. In a trial using maize, Rocha et al. (2019), compared the delivery efficiency of *R. irregularis* via soil inoculation (4860 AM fungal propagules per plant), with seed coating (273 AM fungal propagules per seed), under greenhouse conditions. Results showed a similar root AM colonization between the two inoculation methods, despite the 20-fold difference in the amount of applied inocula. Schoina et al. (2011), in a greenhouse trial, evaluated the biocontrol efficacy of bacterial strain *Paenibacillus alvei* K-165 against the cotton phytopathogenic fungus *Thielaviopsis basicola* using: (1) seeds coated with a K-165 bacterial formulation in 10% xanthan gum-talc, (2) seeds coated with K-165 encapsulated in sodium alginate-Pyrax and (3) solely K-165 encapsulated in sodium alginate-Pyrax pellets. Seed coating with K-165 xanthan gum and talc mixture was the most effective treatment in reducing disease symptoms and increasing plant height and fresh weight compared to sodium alginate-Pyrax encapsulated treatments. This might be due to the fact that coating with a bacterial formulation delivered higher bacterial concentration to the seeds, and consequently to the rhizosphere, in comparison with other methods. In another study, Amutha (2017) compared four different inoculation methods (seed immersion, seed coating, foliar spray and soil drenching) and found that all delivered *B. bassiana* to cotton plants, though with different levels of efficacy. Foliar application followed by soil drenching was considered the most effective inoculation method for *B. bassiana*. Müller and Berg (2008) tested *Serratia plymuthica* inoculation onto canola seeds, using three different techniques (pelleting, film coating and bio-priming), against *Verticillium* dahlia in greenhouse trials. Overall, *Serratia* treated plants had significantly inferior disease severity compared to non-inoculated control, yet the efficiency varied with the employed technique. Film coating resulted in 5.2% disease suppression, while plants treated by pelleting and bio-priming showed 13.4% and 14.3%, respectively. In a field trial conducted by Rehman et al. (2018) *Pseudomonas* sp. MN12 was applied in combination with zinc (Zn) using four different methods (soil application, foliar spray, seed priming and seed coating) to evaluate the interactive effect on wheat productivity. Results revealed that Zn application through any method including seed coating improved grain yield and grain Zn biofortification of bread wheat. Yet, maximum improvement of grain yield was recorded when Zn was applied in combination with strain MN12 through seed priming. The results from the above studies indicate that further investigations comparing different formulations and techniques can contribute to perfect seed coating. Notwithstanding, it is also important to ponder the economic feasibility of the method, since it can compromise large-scale applications.

Agricultural Applications

In general, the application of microbial seed coating in agriculture is aimed at improving crop productivity. Seed coating with PBM has been successfully applied to a wide range of seeds with many different sizes, shapes, textures and germination types (Figure 1.3). The most explored agricultural crops regarding inoculation via seed coating are cereals like wheat and

maize, and fruit/vegetable crops such as tomato, cucumber and sugar beet. Soybean, chickpea and pea are some of most commonly reported oil and seed pulses crops. Additionally, fiber crops like cotton or forage crops like alfalfa have also been addressed in PBM seed coating research.

In most reported studies, application of PBM via seed coating is able to promote crop growth (Sharma et al., 2003; Geetha et al., 2011; Choi et al., 2016; Lally et al., 2017; Rozier et al., 2017; Accinelli et al., 2018b) or biocontrol of phytopathogens (Massoud et al., 2000; Anjaiah et al., 2006; Perelló et al., 2006; Haikal, 2008; Heo et al., 2008; Xue et al., 2013; Ahmed et al., 2016b).

Crop production and nutrition

Not only, seed inoculation can improve plant growth and yield, but also nutritional value of the crops. Recently, Rouphael et al. (2017) evaluated two seed propagated artichoke cultivars 'Romolo' and 'Istar' regarding planting time and seed coating with a consortium of AM fungi (*R. intraradices* and *F. mosseae*) and *T. atroviride*. They found that microbial seed coating improved both plant yield and nutritional value (such as antioxidant activity, total phenolics, caffeoylquinic acids and flavonoids). The results showed that coating seeds with a consortium of PBM could assist host plants to achieve optimal yield with high nutraceutical properties when in combination with appropriate cultivars selection and agronomical practices. The increase in grain yield and yield stability with seed coating treatment was associated with higher nutrient uptake, soil plant analysis development index and photochemical activity of photosystem II. The seed coating formulation with the above mentioned AM fungi and *Trichoderma* consortium was based on previous results reporting enhancement of productivity of winter wheat and vegetable crops. In Colla et al. (2015a) the same consortium was inoculated via seed coating and significantly improved seedling growth (increase of 23, 64 and 29% in shoot and root biomass and the number of leaves, respectively), yield (increase of between 8.3 and 32.1%, depending on the growing season) and grain quality (increase of 6.3% in protein concentrations and general increase in K, P, Fe and Zn concentrations) of winter wheat. When inoculated to the soil in the form of tablets, the same consortium of PBM increased the shoot dry weight (SDW) by 167, 56, 115, 68 and 58% of lettuce, melon, pepper, tomato and zucchini, respectively, in greenhouse experiments, and the shoot and root dry weight of lettuce by 61 and 57%, respectively and the yield of zucchini by 15% under field conditions (Colla et al., 2015b). Seed coating with PBM can be particularly pertinent in low input agriculture, due to its potential to reduce the application of fertilizers and improve food nutritional value. Oliveira et al. (2016a) showed that a silicon dioxide based seed coating was a successful tool to inoculate the AM fungal isolate *R. irregularis* BEG140 that increased dry weight of shoot and seed spikes and nutritional contents (K and Zn) of wheat under reduced fertilization. The same coating formulation was used by Rocha et al. (2019), where maize was grown without fertilization. Single inoculation with *R. irregularis* resulted in shoot

nutrient concentration increments of 110, 93, 88 and 175% for N, P, K and Zn, respectively. In fact, the efficacy of some microbial inoculants for improving plant growth and yield can be influenced by nutrients addition/presence. In the study of Shaharoona et al. (2008), two 1-Aminocyclopropane-1-Carboxylate (ACC)-deaminase producing *P. fluorescens* strains were coated with peat onto wheat seeds. Both pot and field trials revealed that the efficacy of *P. fluorescens* for improving growth and yield of wheat decreased with increasing rates of NPK added to the soil. Results showed that the right combination between proper doses of fertilizer and *P. fluorescens* could be used to improve plant growth while reducing fertilizer application.

Biocontrol

BCA and inducers of systemic acquired resistance (SAR) have been studied in order to reduce the use of fungicides in agricultural crops. Perelló and Bello (2011) evaluated the effectiveness of two *T. harzianum* strains (Th1 and Th2) and two synthetic compounds [acibenzolar-S-methyl (ASM) and thiamethoxam (TM)] on wheat growth and suppression of tan spot caused by the fungal pathogen *Pyrenophora tritici-repentis*. Both biological and chemical agents were considered as SAR inducers. While ASM solution was sprayed on wheat leaves, *Trichoderma* and TM were coated onto seeds. Field trials showed that both biological and chemical agents can generally reduce the severity of tan spot, increasing plant height and weight in comparison with control. Th1 was responsible for reducing the presence of necrotic lesions (>50%), increasing foliar fresh weight (50%) and dry mass (25%). Activation of SAR in plants can be an alternative to maintain crops healthy and vigorous. The right combination of SAR inducers applied via seed coating with reduced rates of appropriate fungicides is a promising option for farmers. Further studies showed that the efficacy of plant disease control of fungicides and BCA applied via seed coating can be comparable. Mahmood et al. (2015) found that in a greenhouse study both fungicides and BCA are almost equally effective against the chickpea wilt pathogen *F. oxysporum*. A treatment combining *T. harzianum* coated onto seeds with 1% methylcellulose solution and soil drench of fungicide carbendazim were proven to be more effective than individual treatment of the fungicide or the biocontrol agent. Mcquilken et al. (1990) showed that coating cress and sugar beet seeds with *P. oligandrum* oospores can control a range of damping-off diseases, in some cases, with the same efficiency as fungicide application. Seed coating with BCA could be used to reduce the amount of fungicide necessary to efficiently suppress disease in a susceptible cultivar. In some cases, the synergetic effect of BCA combined with reduced levels of fungicides can suppress disease equally to a fungicide application at full strength (Howell, 1991). Coating BCA onto agricultural crops can also be a viable, economical and environmentally-friendly strategy for weed control (Elzein et al., 2010). Elzein et al. (2006) showed that coating sorghum seeds with *Fusarium oxysporum* and gum arabic was an effective way to control the root parasitic weed *Striga*. They observed reductions of healthy emerged *Striga* shoots of 81 and 77% in sterilized and non-sterilized soil, respectively.

Abiotic stress tolerance

A small portion of the published research concerning PBM inoculation via seed coating is focused on improving crops resistance to abiotic stress. Recently, Rocha et al. (in press) reported that coating cowpea seeds with *P. putida* using silicon dioxide and starch significantly increased biomass and seed yield under water deficit. The use of microbial inoculants is also considered as a promising option to enhance the production of cereals under salinity stress. Shahzad et al. (2017) showed that seed coating with *Bacillus* spp. improved gas exchange (e.g. photosynthetic rate, transpiration rate and stomatal conductance), ionic content (e.g. N, P and K of grain and straw), biochemical parameters (e.g. chlorophyll, carotenoids and crude protein contents), growth and yield attributes of wheat in saline soils. A greenhouse experiment using chickpea seeds coated with *Paenibacillus lentimorbus* B-30488 in combination with sodium alginate and calcium chloride ($CaCl_2$) increased germination percentage and the number of colony-forming units (CFU) of B-30488 in the rhizosphere, resulting in amelioration of drought stress by positively influencing the dehydration-induced physiological responses (Khan et al., 2011). The study revealed the potential role of sodium alginate and $CaCl_2$ in affecting the biofilm formation of B-30488, and its adequacy for seed coating formulation in stress adaptation and protection of plants under drought stress.

Bio-priming

Bio-priming is a process of biological seed treatment that combines seed hydration and seed inoculation with PBM to accomplish seed protection against soil-borne pathogens improved germination, seedling establishment and vegetative growth (Meena et al., 2017). It is commonly used for biocontrol purposes. The inoculation of PBM in bio-priming can be done either by soaking seeds into a microbial suspension or by seed coating. In a study by Srivastava et al., 2010, tomato seeds were bio-primed by seed coating with inoculum of *T. harzianum* and *P. fluorescens* (either singly or in combination) using a slurry of talc (carrier) and gum arabic (binder). Application of *T. harzianum* and *P. fluorescens* by seed bio-priming significantly decreased the time needed for germination, increased germination rate and reduced the incidence of *Fusarium* wilt in pot and field trials. The combinations of inoculants were more effective than single isolate treatments. Pill et al. (2009) tested non-primed and primed slurry coated cucumber seeds with commercial preparations of *T. harzianum* on seedling emergence and growth in *Phythium aphanidermatum* infested growth medium. While *T. harzianum* coated primed seeds had higher seedling emergence and seedling shoot fresh weight, non-primed *T. harzianum* coated seeds displayed low incidence of damping-off caused by *P. aphanidermatum*. Rao et al. (2009) showed that coating and priming *P. fluorescens* onto sunflower seeds increased the control effect against *Alternaria* blight.

Limitations and inconsistencies

Benefits of microbial seed coating on crop yield can be of short-term or null according to the growing conditions (Kubota et al., 2008). In fact, not all published research shows positive effects on plant performance of PBM inoculation via seed coating. No beneficial effect on crop productivity, nodulation and biological N fixation (Knight, 2007), no economic gains when compared with fungicide application (Hartz and Caprile, 1995) and reduced biocontrol effect (Kay and Stewart, 1994) of inoculated seeds have been reported. For example, Diniz et al. (2009) coated sweet pepper seeds with a mixture of PBM (*Trichoderma viride*, *T. polysporhum*, *T. stromaticum*, *B. bassiana*, *M. anisopliae* and AM fungi) and observed a negative impact on germination rate and plant height. The same undesirable effects were described regarding germination rate of lettuce seeds coated with the same mixture of PBM (Diniz et al., 2006).

Studies on microbial seed coating have been conducted in a similar proportion under laboratory, greenhouse and field conditions (Figure 1.4). Still, only a small number of reports include all scales (e.g. laboratory, greenhouse and field). Inconsistency of field performance can be one of the main restraints for the wide application of seeds coated with PBM. Thus, results that clearly validate the efficacy of the delivery system and the microbial application covering all stages of the process are essential. Shaharoona et al. (2006) tested the effect of ACC-deaminase containing *Pseudomonas* spp. inoculated onto maize via seed dressing on plant growth in pot trials. The most efficient strains in promoting plant height, root weight and biomass of maize were selected and tested under field conditions. Results indicated that rhizobacteria containing ACC-deaminase are effective in improving growth and yield at low levels of fertilizer. Shaharoona et al. (2008) validated the positive effects of ACC-deaminase producing *P. fluorescens* on growth, yield and nutrient use efficiency of wheat under reduced levels of NPK in both pot and field trials. According to Anjaiah et al. (2006), *Pseudomonas* sp., *Bacillus* sp. and *Trichoderma* spp. inoculated onto groundnut via seed dressing were successfully used for biocontrol of pre-harvest seed infection by *Aspergillus flavus* under both greenhouse and field conditions.

The efficacy of microbial application methods may also vary according to the experimental scale. For instance, Kazempour (2004) evaluated the ability of *P. fluorescens* isolates to inhibit *R. solani* in rice under greenhouse and field conditions using different inoculation methods (seed coating, soil drenching and foliar spray). *P. fluorescens* isolates were found to be more effective when delivered via seed coating under greenhouse conditions, while in the field the best results were obtained with seed coating and foliar spray joint application.

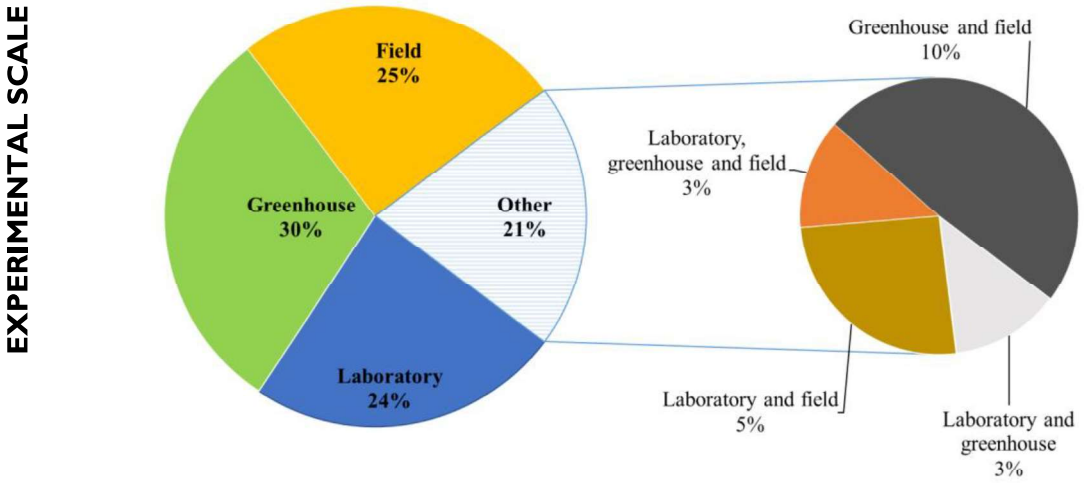

Figure 1.4. Scale of experiments of seed coating inoculation, expressed as percentage of studies (from a total of 191 papers published between 1960 and 2019).

Microbial seed coating is becoming more popular. From 191 studies evaluated, about 41% were developed over the last 9 years. This tendency is in accordance with the growing demand of the global market for biological seed treatment (Markets and Markets, 2018). Figure 1.5 presents the distribution of studies regarding inoculation of PBM via seed coating worldwide. North American countries such as, United States of America and Canada, and from Asia like, India and Pakistan, exhibited the higher number of studies. Nevertheless, Asian and European continents have the biggest increase in research regarding PBM seed coating during the last decade.

Noteworthy, that microbial seed coating market will only reach its potential if bio-inoculants can be produced and applied in a cost-effective way and with efficient functionality regarding the purpose of application. Regardless of the abundant scientific literature on the capacity of several microbial inoculants to improve crop performance and tolerance to abiotic and biotic stresses few of this work has been scaled up to commercial products or properly adapted for large-scale agricultural application.

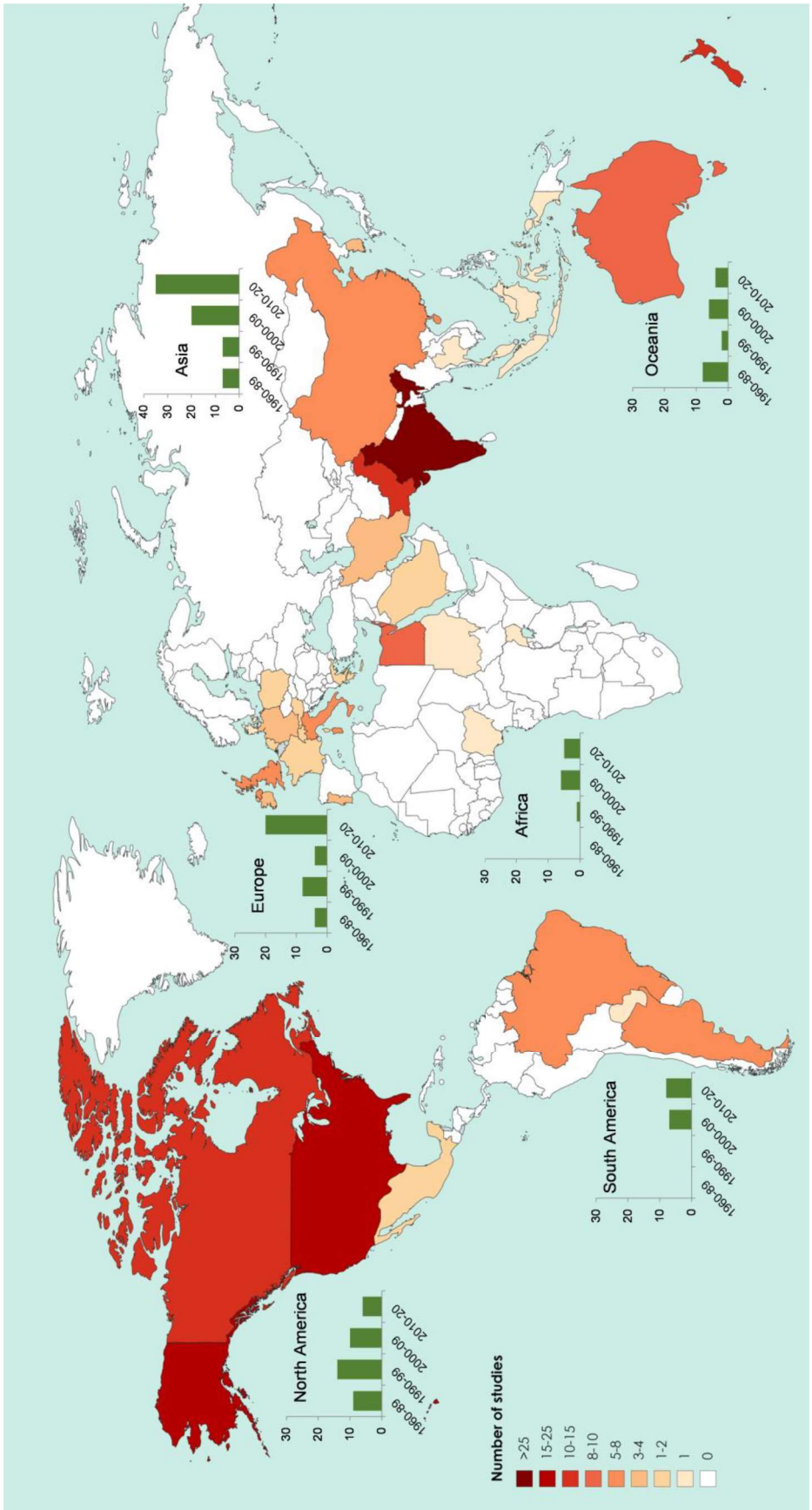

Figure 1.5. World map representing the number of studies dealing with seed coating with plant beneficial microbes by country and continent (from a total of 191 papers published between 1960 and 2019). Charts (green bars) indicate the number of published studies by continent, organized by intervals. Intervals correspond to decades (1990-99, 2000-10, and 2010-20), with the exception of the first interval, which collects studies of a larger period (1960-89) due to the low publication record during this period. Note the different scale of the y axis in the Asia chart.

Research Focus, Objectives and Thesis Outline

The potential of AM fungi and PGPR in agriculture is well recognized (Mäder et al., 2011; Njeru et al., 2015; Oliveira et al., 2017a; 2017b). Yet, despite the benefits that these microbes can bring to crop productivity and quality, their application in large-scale agricultural systems is still limited due to the lack of efficient inoculation methods. Currently, no feasible delivery system for AM fungi application in large-scale agriculture is available (Vosátka et al., 2012; Oliveira et al., 2016b). On the other hand, lack of data on field performance can also be a major limitation for the application of microbial inoculants (Nadeem et al., 2014). Thus, the present thesis intended to address these pitfalls through investigations on seed coating with selected inocula of AM fungi and PGPR in greenhouse and field trials.

In this thesis, the intention was to develop seed coating as an effective delivery system of PBM, contributing to sustainable agriculture. Seed coating with AM fungi and PGPR was expected to significantly decrease the amount of inoculum required for an efficient inoculation. Moreover, consortia of PBM could be more competent than single-strain inocula, due to the various mechanisms of action of the different inoculants (Malusá et al., 2016). Thus, AM fungi and PGPR were inoculated singly and in consortia, which also included the use of single and multiple isolates of AM fungi. The selection of AM fungi and PGPR was intended to increase the efficiency of plant nutrient acquisition, improved tolerance to abiotic stress and enhanced crop yield. Seed coating was proposed to successfully deliver microbial inoculants under greenhouse and field conditions.

Overall, the goals of this thesis were to (1) explore the potential of seed coating as a delivery system of AM Fungi and PGPR for high-value agricultural crops (with seeds of different sizes, shapes and germination types); (2) explore the potential of AM fungi and PGPR to reduce the input chemical fertilizers, improve plant resilience to water deficit and increase crop productivity; and (3) evaluate the effects of microbial inoculation under different experimental scales (greenhouse and field). Specific goal can be found in the following data chapters.

This thesis was organized in six chapters, as shown in Figure 1.6. Chapters 2, 3 and 4 were published, and Chapter 5 submitted for publication as original articles. Chapter 1 is based on a submitted review article (Seed coating: a tool for delivering beneficial microbes to agricultural crops). All articles were published or submitted to international peer-reviewed scientific journals with impact factor.

Chapter 1 comprises a general introduction regarding the importance of agriculture, the need for sustainability, the usefulness of plant-microbes-soil interactions, the role of PGPR and AM fungi as microbial inoculants and the available inoculation methods. Additionally, this chapter includes a review of published research on microbial seed coating types, ingredients, equipment and formulations as well as agricultural applications; ending with the research focus of this thesis as well as proposed goals and structure.

In **Chapter 2**, two greenhouse experiments (A and B) are described. Experiment A was designed to compare the effectiveness of seed coating with direct soil inoculation (conventional inoculation). This included the use of maize seeds without inoculation, inoculated through seed coating or through direct soil inoculation with *R. irregularis* and *Pseudomonas fluorescens* (applied singly or dually). Experiment B, intended to evaluate whether the application of *R. irregularis* and *P. fluorescens* via seed coating could minimize the input of chemical fertilizer in the production of maize. Here, seed germination, growth and nutritional status of plants subjected to different inoculation treatments were assessed under 3 levels of fertilization; no fertilization, reduced fertilization and full fertilization.

Chapter 3 describes a greenhouse experiment that intended to evaluate the performance of cowpea coated with *R. irregularis* and *Pseudomonas putida* under different water regimes. This trial included the combination of four inoculation treatments (non-inoculated seeds, seeds coated with *R. irregularis*; *P. putida* and a mixture of *R. irregularis* + *P. putida)* and three water regimes (no water deficit, moderate water deficit, and severe water deficit). Leaf gas exchange parameters (e.g. steady-state net photosynthesis, stomatal conductance, transpiration rate) and pigments content (chlorophylls and carotenoids) as well as cowpea productivity (e.g. seed yield) and nutritional status were evaluated.

In **Chapter 4** the efficiency of inoculation of single and multiple AM fungal isolates via seed coating and their effects on chickpea productivity under greenhouse and field conditions were assessed. Three inoculations treatments (uncoated and non-inoculated seeds; coated with *R. irregularis;* and with a mixture of *R. irregularis* isolates) were tested. In both field and greenhouse experiments, the impact of the different inoculation treatments in chickpea productivity (e.g. SDW, seed yield, harvest index) and nutritional grain content (e.g. crude protein and fiber, fat) were described. Further, this chapter intended to verify whether results obtained under greenhouse conditions could be an indicator of microbial benefits for field applications.

Chapter 5 presents a field trial where the effects of seed coating with *Pseudomonas libanensis* and *R. irregularis* (single or multiple isolates) on cowpea productivity were evaluated under low-input conditions (fertilization and irrigation). Plant productivity parameters (e.g. seed yield and weight, harvest index), soil properties (e.g. pH, organic matter, N content) and grain nutritional content (e.g. crude protein, fat, ash) were assessed.

Finally, **Chapter 6** includes a summary and brief discussion of the main findings of the previous data chapters. Here, future work lines of microbial seed coating for sustainable agriculture are also presented.

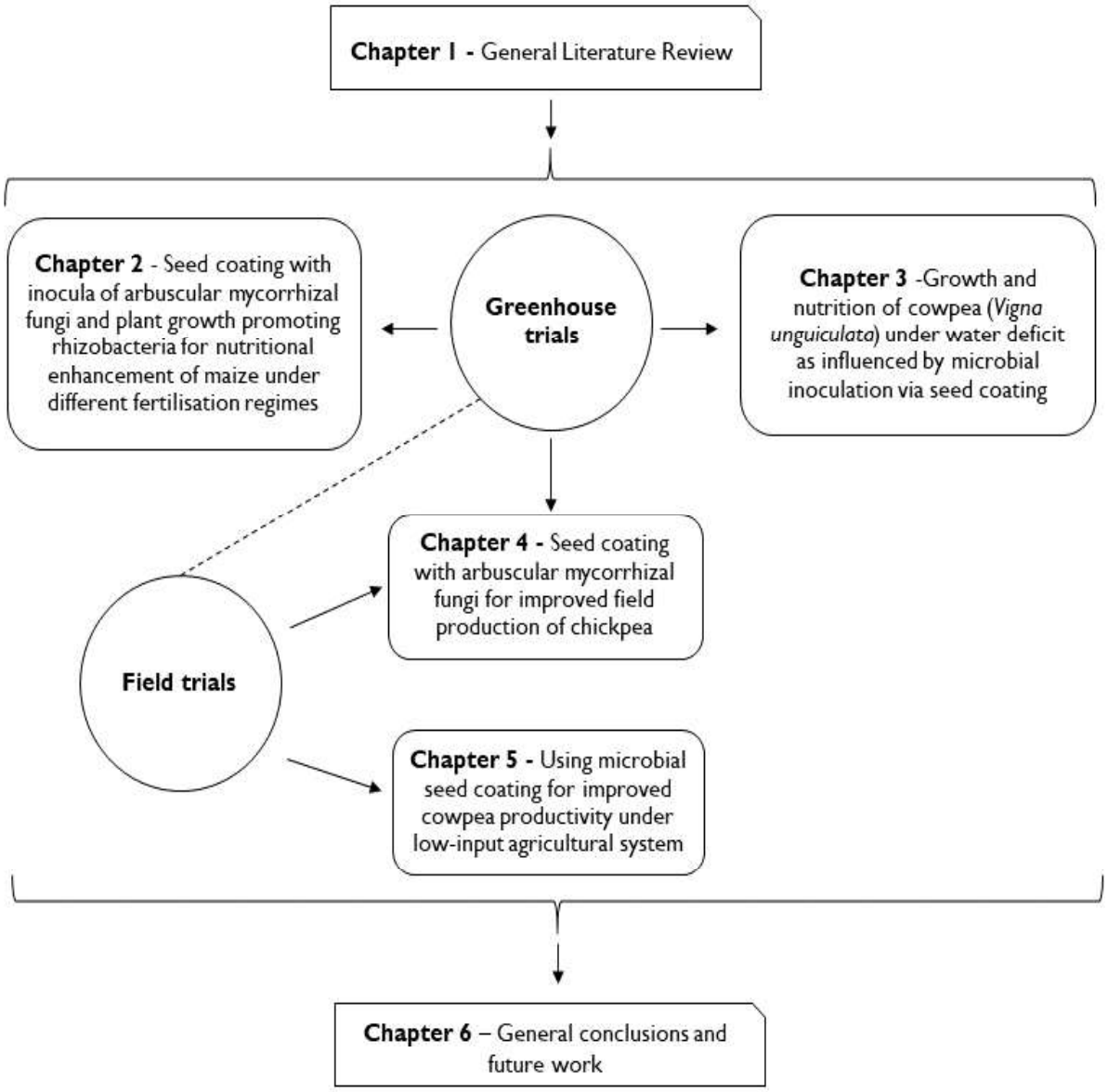

Figure 1.6. Chapters and schematic outline of the PhD thesis

Chapter 2 - Seed coating with inocula of arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria for nutritional enhancement of maize under different fertilization regimes

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This chapter was published as an original article in *Archives of Agronomy and Soil Science*: Rocha I., Ma Y., Carvalho M. F., Magalhães C., Janoušková M., Vosátka M., Freitas H. and Oliveira R. S. (2018). Seed coating with inocula of arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria for nutritional enhancement of maize under different fertilization regimes. *Archives of Agronomy and Soil Science,* 65 (1), 31-43. DOI: 10.1080/03650340.2018.1479061

Abstract

Arbuscular mycorrhizal (AM) fungi and plant growth-promoting rhizobacteria, responsible for enhancing plant nutrition, vigor and growth, may be used to reduce dosages of chemical fertilizers. Technologies that allow an economically viable and efficient application of these beneficial microbes in large-scale agriculture must be studied. Seed coating is a potential delivery system for efficiently introducing minor amounts of bioinoculants. Despite the dramatic reduction on inoculum dose per plant, inoculation of AM fungi via seed coating was as effective as conventional soil inoculation. Fertilization and inoculation had a significant impact on maize shoots nutrient concentrations. Different fertilization regimes did not influence mycorrhizal colonization. Plants without fertilization and singly inoculated with *R. irregularis* showed shoot nutrient concentration increments of 110, 93, 88 and 175% for nitrogen, phosphorus, potassium and zinc, respectively, comparing with non-inoculated controls. Plants singly inoculated with *P. fluorescens* via seed coating under full fertilization, presented enhancements of 100, 75 and 141% for magnesium, zinc and manganese, respectively, comparing with non-inoculated controls. Seed coating is a promising tool for delivering microbial inoculants into the soil, while promoting sustainable production of maize. This technology is particularly pertinent in low input agriculture, with potential environmental profits and food quality improvements.

Keywords: Plant growth promoting microorganisms, Biofertilizers, Soil inoculation, Fertility, Sustainable agriculture

Introduction

A new route on agricultural practices is required to ease the pressure on the environment and human health (Adesemoye and Kloepper, 2009; Malusà et al., 2012). In order to maintain productivity and reduce the input of agrochemicals, the exploitation of plant beneficial microbes, such as arbuscular mycorrhizal (AM) fungi and plant growth-promoting rhizobacteria (PGPR) is of great potential (Kumar et al., 2007; Walker et al., 2011; Couillerot et al., 2013).

The roles of AM fungi in agriculture are widely recognized, as they have the capacity to improve plant fitness by enhancing uptake of nutrients and water, protecting plants against biotic and abiotic stresses and improving soil quality and structure (Mäder et al., 2011; Njeru et al., 2015; Oliveira et al., 2017a; 2017b). On the other hand, PGPR are responsible for promoting growth and plant protection through mechanisms such as production of siderophores and phytohormones, nitrogen fixation, reduction of ethylene levels, solubilization of nutrients and

induction of pathogen resistance (Walker et al., 2011; Bhattacharyya and Jha 2012; Nadeem et al., 2014). Among all the mechanisms they may also stimulate the development of mycorrhiza. Some mycorrhiza helper bacteria, such as *P. fluorescens* F113 can facilitate root colonization by AM fungi, and at the same time display properties of plant growth promoting rhizobacteria (Couillerot et al., 2013).

In agricultural practice only 10 to 40% of the total applied chemical fertilizers are taken by the plants, the remaining is lost by a variety of mechanisms or processes (Bhardwaj et al., 2014). PGPR and AM fungi can greatly improve nutrient use efficiency, leading to a reduced need for chemical fertilizers (Adesemoye and Kloepper, 2009; Bhardwaj et al., 2014; Oliveira et al., 2016a; 2016b).

With over 1 billion ton harvested worldwide in 2013, maize is the world's most cultivated cereal crop, with indubitable economic and nutritional value (Berta et al., 2014; Zerbe, 2015). To meet the growing demand for this cereal and to satisfy the need for a more sustainable agriculture with lower agrochemical inputs, AM fungi and PGPR stand as promising tools (Malusà et al., 2016). Recent studies demonstrated the efficiency of these beneficial microbes in promoting maize growth and yield in field experiments (Adesemoye et al., 2008; Jarak et al., 2012; Krey et al., 2013; Sangeetha et al., 2013; Berta et al., 2014) and in greenhouse trials (Wu et al., 2005; Couillerot et al., 2013). Despite these promising results, the application of both AM fungi and PGPR by broadcasting inocula in open agricultural fields is not economically feasible, since non targeted spreading of inoculum over large areas results in high cost per plant (Vosátka et al., 2012; Oliveira et al., 2016b). In order to use minor amounts of inoculum, seed coating, a technique in which a certain active compound is adhered around the seed, is here proposed as an inoculation mechanism for maize seeds (Colla et al., 2015a; Ehsanfar and Modarres-Sanavy 2004; Oliveira et al., 2016b).

The aims of the present study were to (i) assess the effectiveness of seed coating as a delivery system of inocula of AM fungi and PGPR and (ii) evaluate whether the application of microbial inoculants via seed coating could minimize the input of chemical fertilizer in maize production.

Materials and Methods

Soil and Plant Material

The soil used in this study was a sandy loam with the following properties: 6.5 pH, 0.1 dS m^{-1} electrical conductivity, 1.2% organic matter, 3.8 g kg^{-1} total N, 48.8 mg kg^{-1} extractable P, 4.3 g kg^{-1} K, 1.6 g kg^{-1} calcium (Ca), 66 mg kg^{-1} Mg and 147 mg kg^{-1} sodium (Na). The soil, collected from an organic farm in northern Portugal, was sieved (4 mm) and autoclaved twice at 121 °C for 25 min. Maize (*Zea mays* L.) seeds (ACC N°06694, free pollination) were obtained from

Banco Português de Germoplasma Vegetal, of the National Institute for Agrarian and Veterinary Research (INIAV).

Inoculum Preparation and Seed Coating

The AM fungus used was *R. irregularis* BEG140 grown for 8 months in a multispore pot culture containing a 1:1 (v/v) mixture of zeolite and expanded clay with *Trifolium pratense* L. as host plant. For the seed coating procedure, the *R. irregularis* inoculum was sieved through a 500 µm mesh and mixed with silicon dioxide (1:1 w/w), which served as coating material (the inoculum-coating material mixture was provided by Symbiom Ltd., Czech Republic). For plants where the seeds were not coated, the same AM fungal inoculum was used without sieving.

P. fluorescens F113, a PGPR isolated from sugar beet rhizosphere by Fenton et al., (1992), was purchased from the International Center for Microbial Resources from the Bacteria Associated with Plants strain collection (CFBP 5935) in France (http://www6.inra.fr/cirm_eng/CFBP-Plant-Associated-Bacteria). To obtain *P. fluorescens* inocula, bacteria cells were grown on Luria Bertani (LB) medium supplemented with 0.25 g l^{-1} $MgSO_4 \cdot 7H_2O$ for 8 h at 30 °C and 200 rpm, according to the procedures from Couillerot et al. (2013). For the seed coating, *P. fluorescens* grown in LB media was centrifuged at 7000 rpm for 10 min and resuspended in 10 mM $MgSO_4 \cdot 7H_2O$ with 2% (w/w) glycerol, added as a protective agent to the cell suspension to minimize the loss of cell viability during the coating process, and mixed with the coating material (1:1 v/w). Both fungus and bacterium were also coated together using the same procedure and proportions (1:1:1 w/v/w) as aforesaid. Maize seeds were coated by gradually adding the inoculum-coating mixture and air dried at 22-23 °C for 72 h according to the pan coating method (Scott et al., 1991) as described by Oliveira et al., (2016b). Non-inoculated control seeds were coated only with silicon dioxide.

Experimental Design

This study was divided in two experiments (A and B), which were performed simultaneously. Both trials were conducted in a greenhouse with a temperature and relative humidity (RH) ranging from 14 to 42 °C (average 20 to 30 °C) and from 55 to 85%, respectively, and with an average photoperiod of 12 h. Pots of 3 L were disposed in a fully randomized scheme for both experiments and in order to minimize differences due to their location in the greenhouse, their positions were periodically swapped.

Experiment A aimed at comparing conventional soil inoculation with seed coating inoculation and encompassed seven treatments: (i) non-inoculated controls (control), (ii) *R. irregularis* conventionally inoculated in the soil (RIsoil), (iii) *R. irregularis* inoculated through seed coating (Rcoat), (iv) *P. fluorescens* conventionally inoculated in the soil (PFsoil), (v) *P. fluorescens*

inoculated through seed coating (PFcoat), (vi) a consortium of *R. irregularis* and *P. fluorescens* conventionally inoculated in the soil (RI+PFsoil), and (vii) a consortium of *R. irregularis* and *P. fluorescens* inoculated through seed coating (R+PFcoat). Plants that were treated by conventional soil inoculation with *R. irregularis* (RIsoil), received 12 g of non-sieved inoculum placed 2 cm below one uncoated seed, which corresponded to 4860 AM fungal propagules (viable inoculum) per plant, estimated by the most probable number (MPN) method (Porter, 1979). Pots from the Rcoat treatments received one maize seed coated with *R. irregularis*, which corresponded to 273 AM fungal propagules per plant, estimated by the MPN method after the coating procedure. For the treatment PFsoil, 1 ml of bacterial suspension with a concentration of 10^7 CFU ml^{-1} was pipetted onto each pot that received one uncoated maize seed, while for the coated seed treatment (PFcoat), the same CFU concentration was mixed with the coating material according to the aforementioned procedure. After coating, a final bacterial concentration of 10^5 CFU per coated seed was obtained. The CFU was estimated by placing one coated seed in 1 ml of ringer solution followed by serial dilutions and plate count method. For the treatment RI+PFsoil, each pot received one uncoated seed plus 12 g of fungal inoculum and 1 ml bacterial inoculum as described above. Pots of non-inoculated control plants received one *Z. mays* seed coated only with silicon dioxide. Each treatment combination was replicated 8 times. Each plant received 25 ml of full strength Hoagland solution (composition described below) with 20% of P twice a week.

Experiment B aimed at evaluating the growth and nutritional status of maize inoculated with AM fungi and PGPR via seed coating, under 3 levels of fertilization (no fertilization, reduced fertilization and full fertilization). Experimental pots were arranged in a 4 x 3 factorial design, where the first factor was inoculation [non-inoculated controls (control), *R. irregularis* inoculated through seed coating (Rcoat), *P. fluorescens* inoculated through seed coating (PFcoat) and a consortium of *R. irregularis* and *P. fluorescens* inoculated through seed coating (R+PFcoat)] and the second was fertilization [no fertilization (F0), 80% strength Hoagland solution with 20% of P (F1) and full strength Hoagland solution (F2)]. Fertilized plants received 25 ml of the corresponding Hoagland solution per pot twice per week and non-fertilized plants received 25 ml deionized water. The composition of the full strength Hoagland solution was: 224 mg l^{-1} KNO_3, 235 mg l^{-1} $Ca(NO_3).4H_2O$, 160 mg l^{-1} $NH_4H_2PO_4$, 62 mg l^{-1} $MgSO_4.7H_2O$, 1.77 mg l^{-1} KCl, 0.27 mg l^{-1} H_3BO_3, 0.11 mg l^{-1} $MnSO_4.H_2O$, 0.13 mg l^{-1} $ZnSO_4.7H_2O$, 0.03 mg l^{-1} $CuSO_4.5H_2O$, 0.05 mg l^{-1} H_2MoO_4 (85%MoO_3), 3 mg l^{-1} NaFeEDTA (10% Fe) (Taiz and Zeigher 2002). The reductions of 80% strength and 20% of P were made to the full strength solution. The coating procedure, amounts and concentrations of inocula used in experiment B were the same as those in experiment A. Each treatment combination was replicated 8 times.

AM Fungal Analysis

In both experiments the presence of *R. irregularis* in the roots of maize was assessed by microscopic methods. According to a modified Phillips and Hayman (1970) protocol (Oliveira et

al., 2005), the roots of maize were cut into 1-cm pieces and stained with trypan blue for the assessment of the percentage of root length colonized (RLC) and abundance of arbuscules and vesicles. The RLC % by AM fungi in the mycorrhizal root segments was evaluated by the grid-line intersect method (Giovannetti and Mosse 1980) under a stereomicroscope (Leica EZ4 HD, Germany). Arbuscule and vesicle abundances were examined under a compound microscope (Leica DM 5000-D, Germany) (×100–400) as described by Troulevout et al. (1986) and the percentages determined with the software Mycocalc (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

PGPR Analysis

After 70 days of growth, 1 g of maize roots and adhering soil was sampled and transferred into a 50 ml tube and flash-frozen in liquid N. The extraction of DNA from *P. fluorescens* present in the rhizosphere of maize was performed as described by Couillerot et al. (2010). The samples were homogenized using Precellys24 (Bertin instruments, France) and 250-300 mg used for DNA extraction, using the FastDNA® SPIN® kit for soil (MPBiomedicals, CA, USA). The DNA quantification was made using Qubit fluorometric quantitation system (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. The primers used, F113_1_for (CAAGAAAGGTGAGCCGAGAC) and F113_1_rev (CGACAACCAGCACTTGAGAA) were designed and previously tested, with attainment, for *P. fluorescens* by Von Felten et al. (2010). The quantification by real-time polymerase chain reaction (PCR) was based on the methodology described by Walker et al. (2011). A Step One Plus Real-time PCR system (Applied Biosystems, Canada) was used with the following conditions: 20 µl reaction volume with 0.5 µM of each primer, 2 µl of template DNA and 10 µl Fast Sybr Green mix (Applied Biosystems, Canada). The two-step cycling program included an initial pre-incubation of 20 s at 95 °C followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s.

Plant Analysis

In both experiments (A and B) plants were harvested after a growth period of 70 days, the root system separated from the shoot and washed to remove adhered soil. In experiment B, shoots were dried at 70 °C for 48 h and weighed. After drying, stems were grained and digested according to the European Standard EN 13805 (2014). Total P, K, Ca, Mg, sulfur (S), Fe, manganese (Mn) and Zn were determined by inductively coupled plasma optical emission spectrometry (ICP-OES; GBC Quantima, Australia). Operating conditions for ICP-OES determinations were as follows: 1000 W RF power - 1000 W, 15.0 l min^{-1} plasma gas flow rate, 1.2 l min^{-1} auxiliary gas flow rate, 1.0 l min^{-1} carrier gas flow rate, 50 scan/reading, 3 measurement

replicates and dual detector. Total N was determined with a segmented flow analyzer (Skalar Inc. SanPlus, The Netherlands).

Statistical Analysis

Normality and homogeneity of variances were confirmed and data analyzed using one-way and two-way analysis of variance (ANOVA) for each dependent variable (plant and fungal parameters) versus the independent variables (inoculation, in experiment A and inoculation and fertilization in experiment B). For experiment B the main effects of the factors inoculation (C, PFcoat, Rcoat and R+PFcoat), fertilization (F0, F1 and F2) and their interaction were analyzed. When a significant *F*-value was obtained ($P < 0.05$), treatment means were compared using Duncan's multiple range test. Fungal parameters data were analyzed without including the respective non-inoculated control treatments and the bacteria inoculated treatments. All statistical analyses were performed with the SPSS 23.0.0 software package (IBM SPSS Statistics, USA).

Results

In both experiments, coated and non-coated seeds had a germination rate of 100%.

Efficiency of Seed Coating as an Inoculum Delivery System

In experiment A, after 70 days, non-inoculated plants formed no AM fungal root colonization. All plants inoculated with AM fungi had root mycorrhizal colonization, with values higher than 70% and presence of arbuscules and vesicles (Figure 2.1). The results showed no significant differences in RLC %, arbuscule and vesicle abundances between plants conventionally inoculated in the soil with *R. irregularis* and those inoculated via seed coating, regardless of inoculation with *P. fluorescens* (Figure 2.1). Root length colonization was higher than 60% in all treatments. In experiment B, all AM fungi inoculated plants showed root mycorrhizal colonization, while control and bacteria inoculated treatments presented no AM fungal colonization. The % of RLC, arbuscule and vesicle abundances in the mycorrhizal roots of plants inoculated with *R. irregularis*, presented no significant differences across the different levels of fertilization (Table 2.1). No effect of bacterial inoculation on root colonization by AM fungi was observed in both experiments.

After the coating procedure and prior to sowing, seeds treated with *P. fluorescens* presented a concentration of 10^5 CFU per coated seed, yet after the 70 days of plant growth, it was not possible to detect the bacterial strain by the used molecular methods. Therefore, the presence of *P. fluorescens* could not be confirmed in the roots and rhizosphere of maize.

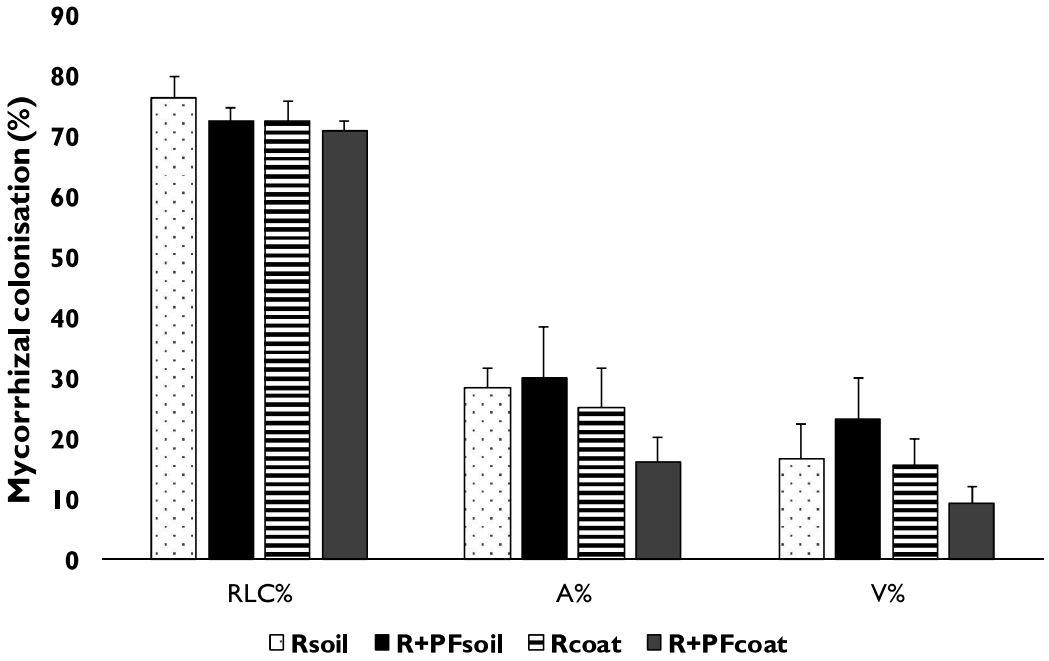

Figure 2.1. Experiment A - Percentage root length colonized (% RLC), arbuscule (A %) and vesicle (V %) abundances in the roots of maize conventionally inoculated in the soil with *Rhizophagus irregularis* (Rsoil) or *R. irregularis* + *Pseudomonas fluorescens* (R+PFsoil) or inoculated via seed coating (Rcoat and R+PFcoat). Values are means ± 1 SE. There were no significant differences according to Duncan's Multiple Range test at $P < 0.05$.

Table 2.1. Experiment B - Percentage of root length colonized (% RLC), arbuscule (A %) and vesicle (V %) abundances of *Rhizophagus irregularis* (Rcoat) and *R. irregularis* and *Pseudomonas fluorescens* consortium (R+PFcoat) inoculated via seed coating in the roots of maize under no fertilization (F0), reduced fertilization (F1) and full fertilization (F2).

Inoculation	Fertilization	RLC (%)	A (%)	V (%)
	F0	67.8 ± 3.8	25.9 ± 8.6	18.3 ± 8.8
Rcoat	F1	62.2 ± 6.3	17.7 ± 6.3	10.7 ± 3.0
	F2	69.7 ± 6.6	25.5 ± 4.3	15.1 ± 3.1
	F0	64.5 ± 1.6	19.3 ± 4.1	6.3 ± 1.6
R+PFcoat	F1	76.8 ± 1.1	16.8 ± 3.1	12.4 ± 2.0
	F2	62.4 ± 5.6	17.1 ± 5.8	9.5 ± 2.8

Values are means (±1 SE). There were no significant differences according to Duncan's Multiple Range test at $P < 0.05$.

Growth and Nutritional Status of Maize Inoculated via Seed Coating

In experiment B, both shoot and root dry weights of maize were positively affected by the fertilization regime. For instance, roots and shoots had higher biomass at full fertilization and lower biomass without fertilization, irrespective of the inoculation treatments (Figure 2.2 and Table 2.2). Inoculation had a significant impact on root biomass and no influence on shoots, being the interaction between inoculation and fertilization only significant regarding roots (Table 2.2). Plants subjected to reduced fertilization and inoculated with *R. irregularis* presented lower root biomass when compared with the remaining treatments. Overall, plants inoculated with beneficial microbes showed no growth enhancement. Both inoculation and fertilization factors influenced the final maize shoot nutrient concentrations (Tables 2.3 and 2.4). Plants inoculated singly with *P. fluorescens* (PFCoat) increased their shoot concentration of N, K, Ca, Mg and Mn by 40, 49, 60, 100 and 141%, respectively. Most of the increments were observed under full fertilization regime. Treatments where only *R. irregularis* was added (Rcoat), showed substantial increases in N and Zn shoot concentrations under all fertilization levels. Nevertheless, the higher values of enhancement and the number of nutrients affected by the AM fungi inoculation where obtained in the F0 and F1 fertilization levels.

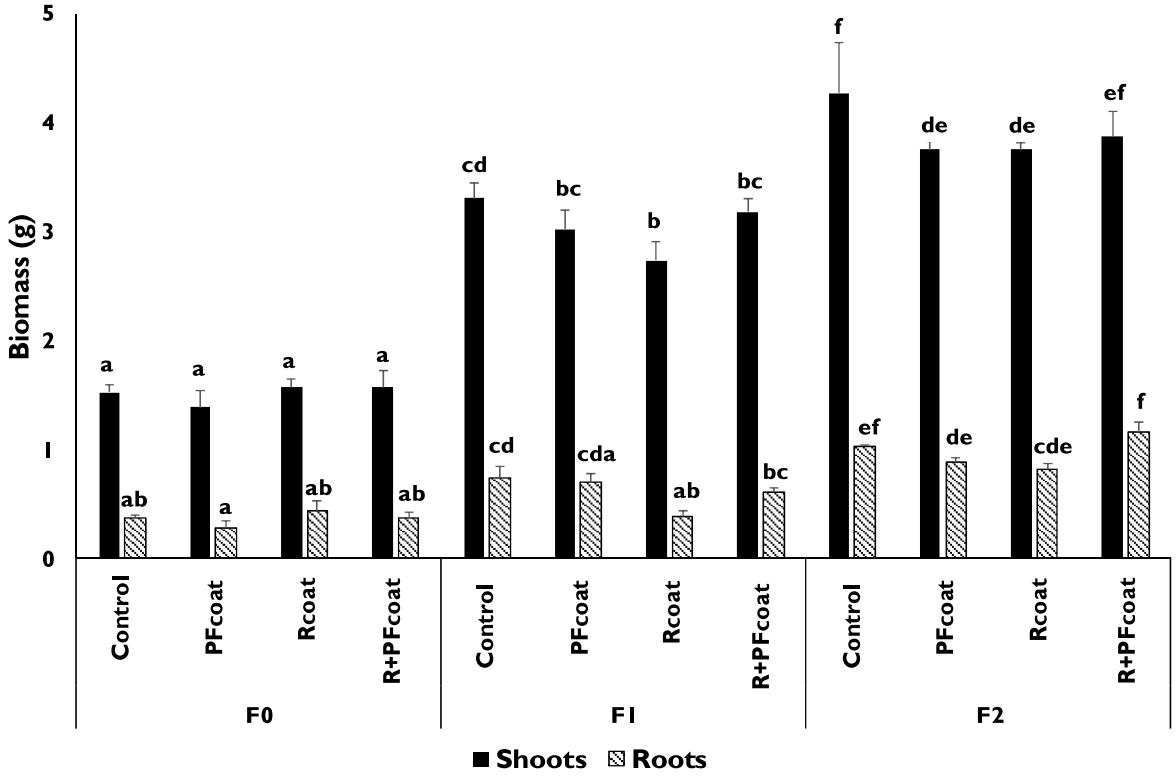

Figure 2.2. Experiment B - Shoot and root dry weight of maize obtained from coated seeds non-inoculated (control), inoculated with *Rhizophagus irregularis* (Rcoat), *Pseudomonas fluorescens* (PFcoat) and a consortium of *R. irregularis* and *P. fluorescens* (R+PFcoat) under no fertilization (F0), reduced fertilization (F1) and full fertilization (F2). Values are means (±1 SE) followed by letters that indicate significant differences according to Duncan's Multiple Range test at $P < 0.05$.

In Rcoat treatment without fertilization (F0) N, P, K, Mg and Zn had increments of 110, 93, 88, 73 and 175%, respectively. In reduced fertilization regime (F1) the same nutrients had increases of 44, 20, 68, 58 and 145%, respectively, while under full fertilization (F2) only N and Zn concentrations were enhanced. Plants inoculated with *R. irregularis* + *P. fluorescens* (R+PFcoat) showed a significant enhancement of N, Ca, Mg and Zn shoot concentration, mainly under F0 and F1 fertilization levels. It is noteworthy that in plants inoculated with AM fungi, nutrient content enhancement was higher in treatments under reduced fertilization than in those under full fertilization. No influence by *R. irregularis* or *P. fluorescens* was noticed in S and Fe shoot concentrations. The analyses of the main effects of microbial inoculation on maize shoot nutrient concentration showed significant increases in all assessed nutrients, except for P and S in PFcoat, Fe and Mn in Rcoat and K, S and Fe in R+PFcoat (Table 2.4). The main effects of fertilization only showed significant differences in N and P shoot concentrations. Higher fertilization resulted in increased N and reduced P shoot concentration (Table 2.4).

Table 2.2. Experiment B - Main effects of the factors inoculation and fertilization and two-way ANOVA *F*-values and significances for shoot and root biomass of maize.

Main effects		SDW (g)	Root dry weight (g)
Inoculation (I)	Control	2.74 a	0.63 b
	PFcoat	2.61 a	0.61 ab
	Rcoat	2.51 a	0.50 a
	R+PFcoat	2.77 a	0.62 ab
Fertilization(F)	F0	1.52 a	0.37 a
	F1	3.06 b	0.61 b
	F2	3.88 c	0.95 c
Two-way ANOVA F-values and significances			
Inoculation (I)		2.7 ns	3.5*
Fertilization (F)		218.1***	60.2***
I x F		1.1 ns	3.4*

Letters indicate significant differences according to Duncan's Multiple Range test. * and ***, significant effect at the level of $P < 0.05$ and $P < 0.001$, respectively; ns, non-significant effect. Control, non-inoculated control; PFcoat, *Pseudomonas fluorescens;* Rcoat, *Rhizophagus irregularis*; R+PFcoat, consortium of *R. irregularis* and *P. fluorescens*; F0, no fertilization; F1, reduced fertilization; F2, full fertilization. SDW, shoot dry weight.

Table 2.3. Experiment B - Shoot nutrient concentrations of *Zea mays* L. under different inoculation treatments via seed coating [Non-inoculated controls (C), *Rhizophagus irregularis* (Rcoat), *Pseudomonas fluorescens* (PFcoat) and a consortium of *R. irregularis* and *P. fluorescens* (R+PFcoat)] and fertilization regimes [no fertilization (F0), reduced fertilization (F1) and full fertilization (F2)].

Inoculation	Fertilization	N (g kg^{-1})	P (g kg^{-1})	K (g kg^{-1})	Ca (g kg^{-1})	Mg (g kg^{-1})	S (g kg^{-1})	Fe (mg kg^{-1})	Zn (mg kg^{-1})	Mn (mg kg^{-1})
Control	F0	8.4±0.4 a	3.0±0.8 ab	35.6±8.4 a	2.7±0.9 a	1.1±0.3 ab	1.0±0.1 ab	71.2±9.1 ab	24.0±5.7 ab	58.4±10.2 a
	F1	12.7±1.3 bc	2.0±0.5 a	39.6±2.1 ab	2.2±0.8 a	1.2±0.2 abc	0.9±0.2 a	49.1±10.4 a	17.2±4.4 a	73.1±29.1 ab
	F2	12.6±1.6 bc	3.1±0.4 ab	39.6±3.1 ab	2.5±0.7 a	1.0±0.1 a	1.1±0.3 ab	64.7±6.6 ab	24.4±2.9 ab	60.2±7.4 ab
PFcoat	F0	11.1±0.6 ab	3.6±0.0 ab	56.0±4.0 bcd	5.1±0.6 abc	1.5±0.0 abcd	1.2±0.0 ab	108.2±42.1 ab	36.4±2.5 ab	86.8±7.1 abc
	F1	13.4±1.2 bc	2.9±0.4 ab	48.4±3.1 abc	6.8±1.3 c	1.7±0.3 abcd	1.3±0.2 ab	90.7±20.1 ab	34.7±6.2 ab	134.0±11.5 cd
	F2	17.6±1.0 d	3.2±0.3 ab	58.9±5.9 cd	4.0±0.6 abc	2.0±0.0 d	1.5±0.2 ab	116.2±19.2 b	42.6±6.9 bc	145.3±25.8 d
Rcoat	F0	17.6±2.1 d	5.8±0.3 c	67.1±7.6 d	3.4±0.6 ab	1.9±0.2 d	1.5±0.1 ab	83.2±4.1 ab	66.0±12.1d	70.2±6.6 ab
	F1	18.3±0.8 d	3.6±0.4 ab	66.5±2.0 d	4.7±0.7 abc	1.9±0.1 d	1.3±0.4 ab	80.3±17.8 ab	42.2±7.3 bc	58.9±13.2 a
	F2	18.1±1.3 d	3.5±0.4 ab	56.8±3.8 bcd	4.6±0.6 abc	1.8±0.2 cd	1.7±0.3 b	83.0±19.1 ab	46.0±4.1 cd	57.4±5.3 a
R+PFcoat	F0	15.3±0.6 cd	3.7±0.2 ab	39.4±5.7 ab	3.8±0.5 abc	1.9±0.0 d	1.1±0.3 ab	62.9±11.9 ab	54.0±19.9 cd	96.0±20.9 abcd
	F1	16.7±1.7 cd	4.1±0.3 ab	39.3±5.8 ab	6.3±1.9 bc	1.7±0.3 abcd	1.5±0.1 ab	60.8±19.0 ab	55.7±9.8 cd	103.8±10.1 abcd
	F2	16.4±1.4 cd	3.2±0.6 ab	57.1±8.4 bcd	4.3±0.8 abc	1.7±0.3 bcd	1.1±0.2 ab	57.0±8.6 ab	36.6±1.5 ab	113.9±23.4 bcd

Values are means (±1 SE) followed by letters that indicate significant differences according to Duncan's Multiple Range test at $P < 0.05$.

Table 2.4. Experiment B - Main effects of the factors inoculation and fertilization and two-way ANOVA *F*-values and significances for shoot nutrient concentrations of *Zea mays* L.

Main Effects		N (g kg^{-1})	P (g kg^{-1})	K (g kg^{-1})	Ca (g kg^{-1})	Mg (g kg^{-1})	S (g kg^{-1})	Fe (mg kg^{-1})	Zn (mg kg^{-1})	Mn (mg kg^{-1})
Inoculation(I)	Control	11.2 a	2.7 a	38.3 a	2.5 a	1.1 a	1.0 a	61.7 a	21.8 a	63.9 a
	PFcoat	14.0 b	3.3 ab	54.4 bc	5.3 b	1.7 b	1.3 ab	105.1 b	37.9 b	122.1 b
	Rcoat	18.0 c	4.3 c	63.4 c	4.3 b	1.9 b	1.5 b	82.2 ab	51.4 c	62.2 a
	R+PFcoat	16.1 bc	3.7 bc	45.3 ab	5.3 b	1.8 b	1.2 ab	60.4 a	48.8 bc	104.6 b
Fertilization (F)	F0	13.1 x	4.0 y	49.5 x	3.8 x	1.6 x	1.2 x	81.7 x	45.1 x	77.9 x
	F1	15.2 *y*	3.2 x	48.5 x	5.1 x	1.6 x	1.2 x	70.2 x	37.5 x	92.5 x
	F2	16.2 *y*	3.3 x	53.1 x	3.9 x	1.6 x	1.3 x	80.2 x	37.4 x	94.2 x
Two-way ANOVA *F*-values and significances										
Inoculation (I)		15.5***	7.6***	12.1***	5.5*	8.7***	3.4*	3.9*	11.1***	10.1***
Fertilization (F)		6.2 ***	5.1*	0.8 ns	2.5 ns	0.0 ns	0.6 ns	0.5 ns	1.6 ns	1.2 ns
I x F		1.6 ns	2.9*	1.7 ns	0.9 ns	0.7 ns	0.8 ns	0.2 ns	1.6 ns	1.1 ns

Letters indicate significant differences according to Duncan's Multiple Range test. * and ***, significant effect at the level of $P < 0.05$ and $P < 0.001$, respectively; ns, non-significant effect. C, non-inoculated controls; PFcoat, *Pseudomonas fluorescens*; RIcoat, *Rhizophagus irregularis*; RI+PFcoat, consortium of *R. irregularis* and *P. fluorescens*; F0, no fertilization; F1, reduced fertilization; F2, full fertilization.

Discussion

The seed coating process used in this study had no negative effect on seed germination. Previously, the same seed coating method had been used with wheat seeds by Oliveira et al. (2016b), also with a germination rate of 100%. Maize and wheat seeds have different sizes and shapes, showing the applicability of this seed coating procedure to dissimilar types of seeds. Due to the relatively high cost of AM fungi inocula per plant, the application in open agricultural fields, apparently is not economically feasible (Vosátka et al., 2012). This study showed that in the case of AM fungi, the use of minor amounts of inoculum through inoculation via seed coating is possible, resulting in similar root colonization when compared with conventional soil inoculation. Comparable results were also obtained by Oliveira et al. (2016b) with wheat seeds coated with AM fungi. With the seed coating process, inoculated bacteria can suffer a loss of viability in the seed, which consequently could have a negative effect on colonization and persistence of bacteria in the soil (Pedrini et al., 2017). However, after the coating procedure and prior to sowing, seeds treated with *P. fluorescens* presented a concentration of 10^5 CFU per coated seed, which is sufficient for successful colonization (Weller, 1983; Tang et al., 1995; Landa et al., 2003). Yet, after the 70 days of plant growth, it was not possible to detect the inoculated bacterial strain in the soil samples by molecular methods, indicating that the concentration of *P. fluorescens* was possibly below the detection limit for the real-time PCR analysis. That fact might be related with the findings of Von Felton et al. (2010) who reported a decrease with time in the population density of *P. fluorescens* F113. This was also pointed out by Haas and Défago (2005), who showed that introduced PGPR can colonize plant roots initially at levels of about 10^7–10^8 CFU g^{-1} but these levels always decline in a few weeks. The persistence in the soil of introduced rhizobacteria can vary considerably from plant to plant (Landa et al., 2003). The decline can be related with several factors such as direct growth inhibition, resource competition (root exudates utilization) or need of a wider range of resources than other bacteria (Adee et al., 1990; Farrar et al., 2014). Moreover, in experiments with wheat and maize, Rosas et al. (2009) showed that *Pseudomonas aurantiaca* can, in fact, decrease over time in rhizosphere soil yet, effects of the inoculated bacteria on plant growth were shown during the whole cycle of the crop. Thus, in our study, the fact that the presence of *P. fluorescens* F113 in the soil could not be confirmed after 70 days, should not lead to the conclusion that there was no bacterial effect in different phases of plant development. In future studies, it will be crucial to perform time course samplings throughout the development of the roots in order to understand the behavior of the inoculated bacteria and also to comprehend the impact on the target plants of changes in bacterial concentrations in the roots and rhizosphere. Depending on the bacteria, the development of mycorrhiza can be negatively or positively affected. In fact, most of these interactions are competitive, but some can be beneficial to the mycorrhizal colonization process (Garbaye, 1994). *P. fluorescens* are of great predisposition to benefit

mycorrhiza establishment and specifically strain F113 proved to be capable of improving the formation of AM associations, as previously shown for other rhizosphere microorganisms (Barea et al., 1998). However, the stimulatory effects by *P. fluorescens* on AM fungi root colonization was not noticed in the present study, since no difference in maize mycorrhizal colonization was observed.

Maize has a high demand for N and P and their soil concentrations can affect AM fungal development. In fact, AM fungal colonization is often negatively correlated with soil P values (Gianinazzi and Schüepp, 1994; Liu et al., 2000). However, the results showed no significant difference in AM fungal colonization between the treatments under different fertilization regimes. P is critical for maximizing plant growth and crop yields, playing a key role in several plant functions and making up to about 0.2% of the dry weight (Smith et al., 2011). Consequently, its absence or low amounts have negative consequences for plant development. The 80% decrease of P instead of 20% of the remaining nutrients, in the treatment of reduced fertilization (F1), might contributed to hamper maize growth. Both AM fungi and PGPR are extensively recognized for their role in agriculture as biofertilizers (Vessey, 2003; Nadeem et al., 2014). Nonetheless, the application of PBM may not always contribute to plant growth, having other beneficial effects on plants such as nutritional enhancement (Ryan and Graham 2002). Even so, increases in plant nutrient concentration may not always translate into enhanced growth and yield (Miller, 2000; Galvez et al., 2001). In this study no enhancement of biomass on plants inoculated with beneficial microbes was observed, being plant growth mainly dictated by the fertilization regime. Although, no significant improvement in plant growth was observed, there was a noteworthy augmentation on nutrient shoot content by microbial inoculation including in the reduced (F1) or no fertilization (F0) regimes. Plants require both macro and micronutrients which are generally obtained from the soil (White and Brown, 2010). AM fungal roots can greatly enhance acquisition of mineral nutrients in host plants, especially those that are of low mobility or sparingly soluble (Clark and Zeto 2000). The N, P, K, Mg and Zn content on maize were positively affected by inoculation with *R. irregularis*, particularly in treatments where fertilization was reduced or absent. The uptake of micronutrients by mycorrhizal plants is considered to be negatively influenced by the availability of P in the soil, which might explain the effect of mycorrhizal plants on Zn content (Lambert et al., 1979; Liu et al., 2000). The significance of AM fungi inoculation might be highest at low nutrient availability, mainly P. In fact, even though no difference in mycorrhizal colonization was observed, the efficiencies of AM fungi in increasing shoot nutrient concentrations varied according to the fertilization regime. N shoot concentration was directly correlated with fertilization, and this might be related with the high demand of N by maize plants (Schröder et al., 2000). On the other hand, plants with single *P. fluorescens* inoculation presented most of the nutritional increments when full fertilization was applied. This might indicate that the beneficial properties of the bacteria are

stimulated by the presence of higher levels of nutrients in the soil. These results supported the overall hypothesis that microbial inoculum can increase nutrient assimilation of plants and can be used for integrating nutrient management strategies (Alloush and Clark 2001; Wu et al., 2005; Adesemoye et al., 2008; Berta et al., 2014). This ability in enhancing the concentration of nutrients provides an added value to food plants, which currently should be taken in great consideration.

Conclusions

The exploitation of beneficial microbes as biofertilizers appears to be a natural route. Particularly in low agrochemical input systems, they can be responsible for maintaining long term soil fertility and sustainability by improving the uptake efficiency and availability of macro and micro nutrients to plants. Plants inoculated with AM fungi and PGPR via seed coating displayed enhanced shoot concentration of macro and micronutrients, under different fertilization regimes. The increments of maize nutrient contents suggest that PBM-based inoculants applied via seed coating can be used and should be further evaluated as component of integrated nutrient management strategies. To our knowledge this is the first report on successful coating of maize seeds with inocula of AM fungi and PGPR. Seed coating for AM fungi inoculation had the same efficiency as direct soil inoculation, showing that there is great potential for PBM inoculation in large-scale agriculture, as it can allow the use of minor amounts of inocula and a more precise application. Thus, seed coating can open the way for large-scale inoculation of beneficial microorganisms in maize production. Additionally, field experiments with maize and other crops will be useful to verify the efficacy of seed coating as a microbial delivery system and the benefits of the application.

Chapter 3 - Growth and nutrition of cowpea (*Vigna unguiculata*) under water deficit as influenced by microbial inoculation via seed coating

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Abstract

Drought can drastically reduce cowpea biomass and grain yield. The application of plant growth-promoting rhizobacteria and arbuscular mycorrhizal fungi can confer resistance to plants and reduce the effects of environmental stresses, including drought. Seed coating is a technique which allows the application of minor amounts of microbial inocula. Main effects of the factors inoculation and water regime showed that: severe or moderate water deficit had a general negative impact on cowpea plants; total biomass production, seed weight and seed yield were enhanced in plants inoculated with *P. putida*; inoculation of *R. irregularis* significantly increased N and P shoot concentrations; and *R. irregularis* enhanced both chlorophyll b and carotenoids contents, particularly under severe water deficit. Plants inoculated with *P. putida* + *R. irregularis* had an increase in shoot P concentration of 85% and 57%, under moderate and severe water deficit, respectively. Singly inoculated *P. putida* improved potassium shoot concentration by 25% under moderate water deficit. Overall, in terms of agricultural productivity the inoculation of *P. putida* under water deficit might be promising. Seed coating has the potential to be used as a large-scale delivery system of beneficial microbial inoculants.

Keywords: Arbuscular mycorrhizal fungi, Plant growth-promoting bacteria, Seed inoculation

Introduction

The agriculture sector is facing a real challenge against climate change (Vurukonda et al., 2016). With the increase in heat waves, storms, droughts, floods or heavy precipitation, crop productivity and food security are being endangered (Hansen et al., 2012; Sundström et al., 2014). Among these climate change threats, drought is expected to dramatically hamper plant growth and development for more than 50% of the arable lands by 2050, decreasing crop productivity worldwide (Kasim et al., 2013; Li et al., 2014). From moderate and short to extremely severe and prolonged periods, drought can disturb plant water potential and turgor and thus modify physiological and morphological traits of plants (Rahdari and Hoseini, 2012). Some beneficial soil microorganisms can help plants overcome problems caused by abiotic stress (Bardi and Malusà, 2012; Bhardwaj et al., 2014; Egamberdieva and Adesemoye, 2016; Vassilev et al., 2015). The exploitation of plant beneficial microbes, such as plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal (AM) fungi for drought stress mitigation in plants, is gaining importance (Li et al., 2014; Nadeem et al., 2014; Vurukonda et al., 2016). Besides their contribution to nutrient acquisition and biocontrol, PGPR can also confer drought tolerance in plants by osmotic adjustment, antioxidant metabolisms and phytohormone modulation (Rubin et al., 2017;

Vurukonda et al., 2016). AM fungal symbiosis can improve plant antioxidant activity, osmotic regulation, photosynthetic rates and pigments, root water absorption and transport and uptake of nutrients, especially phosphorus (P) (Li et al., 2014; Oliveira et al., 2016a; 2016b; Quiroga et al., 2017).

Grain legumes are important for a variety of reasons, since they are a significant and cheap source of protein, are able to fix N in agricultural ecosystems and can be used for industrial and medicinal purposes (Farooq et al., 2017). Cowpea is an important seed crop legume for human consumption (seeds and pods) and for soil amendment and fertilization (e.g. green manure and organic material) (Manaf and Zayed, 2015). Plant biomass and grain yield of legumes can be seriously hampered by moderate to severe drought stress (Farooq et al., 2017). Inoculation with AM fungi and PGPR has been considered to be a promising strategy to increase plant drought tolerance (Bhardwaj et al., 2014; Dodd and Ruiz-Lozano, 2012). Some studies presented the effects of beneficial microbes on plant under water stress, such as improved grain yield and protein content (Oliveira et al., 2017a, b) increment on nutrient (Ngakou et al., 2007) and water uptake and increased transpiration and photosynthesis rates (Virakornphanich et al., 1994). Therefore, it is imperative to develop feasible strategies for application of these beneficial microbes in open agricultural fields using minor amounts of inoculum for precision agriculture. Seed coating is a process where exogenous materials are applied to the surface of the seed and can be used for delivering active ingredients, including beneficial microbes (Pedrini et al., 2017). This technique intends to use minor amounts of inocula in a more precise application that should be as efficiently as conventional soil inoculation. Seed coating could serve as a powerful tool for large-scale inoculation of beneficial microorganisms (Oliveira et al., 2016b).

The main goal of the present study was to assess the impact of the application of PGPR and AM fungi via seed coating in cowpea production under water stress.

Materials and Methods

Seeds and Soil Material

Seeds of cowpea [Vigna unguiculata (L.) Walp. cv. Fradel] were used in this study. The soil used in the experiment presented a loam texture with pH (1:2.5 w/v water) 7.1, electrical conductivity 0.045 dS m^{-1}, 0.16% organic matter, 0.11 g kg^{-1} total N, 3,542 mg kg^{-1} extractable (Egner-Riehm) P and 13 mg kg^{-1} K. Previous to use the soil was sieved through a 4-mm mesh and autoclaved twice (121°C for 25 min) on consecutive days.

Microbial Inocula and Seed Coating

The AM fungus used was *R. irregularis* PH5 grown for 8 months in a multispore pot culture containing a 1:1 (v/v) mixture of zeolite and expanded clay with *Zea mays* L. as host plant.

Regarding the seed coating procedure, the *R. irregularis* inoculum was sieved through a 500-µm mesh and mixed with starch/silicon dioxide mixture (coating material) in the proportion of 1:1 (w/w) (the inoculum-coating material mixture was provided by Symbiom Ltd., Czech Republic). *P. putida* strain GP was isolated from an agricultural soil in central Portugal used to grow *Lupinus albus* L. and tested positively for indoleacetic acid (IAA) (Brick et al., 1991), ammonia (Cappuccino and Sherman, 1992) and siderophores production (Schwyn and Neilands, 1987), phosphate solubilization (Gaur, 1990), N fixation (Dobereiner et al., 1976), biofilm formation in the presence of different salt concentrations, 0.5 to 2.5 M (Christensen et al., 1985) and water stress tolerance (Ma et al., 2016). For the seed coating with bacteria, P. putida was grown in LB media for 17 hr at 28–30°C and 150 rpm, centrifuged at 3,500 rpm for 15 min and re-suspended in ringer solution with 1% carboxy methylcellulose (as an adhesive agent). The bacterial suspension at a concentration of 10^8 CFU ml^{-1} was mixed with the coating material (1:1 v/w). Both AM fungus and bacterium were also coated together using the same procedure and proportions (1:1:1 w/v/w) as aforesaid. For seeds coated with *R. irregularis*, the AM fungal propagules per seed estimated by MPN were 21 (Porter, 1979). Cowpea seeds were coated by the pan coating method (Scott et al., 1991) as described by Oliveira et al. (2016). Non-inoculated control seeds were coated only with the starch/silicon dioxide mixture.

Experimental Design

This study was conducted in a heated greenhouse (temperature ranging from 18 to 30°C) with an average photoperiod of 12 hr using pots of 2 L disposed in a fully randomized scheme. Each pot received 1 seed. The positions of the pots were periodically swapped to minimize differences caused by their location in the greenhouse. All pots received 50 ml of microbial populations filtrate (Whatman No. 1 filter) from the original non-sterile soil as described by Oliveira et al., (2006), in order to provide a common soil microbiota for all the treatments. The experimental design involved twelve treatments, resulting from the combination of four inoculation treatments via seed coating [non-inoculated control (control); plants inoculated with *R. irregularis* PH5 (RIcoat); *P. putida* (PPcoat) and a mix of *R. irregularis* + *P. putida* (MIXcoat)] and three water regimes [no water deficit, 80–75% of water holding capacity (D0); moderate water deficit, 60–55% of water holding capacity (D1); and severe water deficit, 30–25% of water holding capacity (D2)]. Each treatment had six replicates. During the first 3 weeks of plant growth, water was supplied daily to reach 80% of water holding capacity in all treatments. Volumetric soil moisture was measured with a ML2x ThetaProbe (AT Delta-T Devices Ltd, Cambridge, UK), where changes in the apparent dielectric constant of moist soil allowed measuring the volumetric soil moisture content (Roth et al., 1992; White et al., 1994). Before starting the experiment, measures were performed to match the water holding capacity of the soil with the volumetric soil moisture. The 100%, 85–80%, 60–55% and 30– 25% of soil water holding capacity corresponded to 22, 16, 10–9 and 6–5%

volumetric soil moisture, respectively. In order to control water deficit and maintain it at the desire level, the soil water content was measured daily with the ThetaProbe ML2x at the end of the afternoon (5:00–6:00 p.m.) and the amount of water lost was added to each pot. For fertilization, each plant received 20 ml of modified white mineral solution P2N3 (Gryndler et al., 1992) twice a week.

Gas Exchange Parameters

The steady-state net photosynthesis A (Pn), stomatal conductance (gs), intercellular CO2 concentration (Ci) and transpiration rate (Tr) were determined using a Li-6400 IRGA (LI-COR, Lincoln, NE, USA). A 300 µmol s^{-1} flow of non-contaminated air was provided to the leaves using a leaf chamber and mass flow controllers. The analyzed leaves were exposed to a saturating photosynthetic photon flux density of 1000 µmol m^{-2} s^{-1}, block leaf temperature of 25°C and with the RH of the air within the apparatus ranging between 45 and 55%. In all cases, only mature, fully expanded leaves were selected for measurements from four different plants of each experimental condition. The measurements for gas exchange were recorded between the late morning (9:00–11:00 a.m.) and early afternoon (1:00–3:00 p.m.). The instantaneous water use efficiency (WUE) (µmol CO_2 per mmol H_2O) was calculated by dividing the values of steady-state net photosynthesis by the transpiration rate (Pn/Tr).

Chlorophylls and Carotenoids Content

Fresh cowpea leaves (about 0.2 g) were homogenized in chilled N, N-dimethylformamideand stored overnight in the dark at 4°C (Moran and Porath, 1980). The absorptions were measured at 664, 647 and 461 nm using a HACH DR/4000U spectrophotometer (HACH Company, Loveland, CO, USA). Chlorophyll a and chlorophyll b were estimated using the equations of Inskeep and Bloom (1985) and carotenoids using the equation of Chamovitz et al., (1993).

Biomass Production, Seed Yield and Nutrients Acquisition

At harvest, pods were separated and weighted to determine fresh weights. After recording the weight of pods, seeds were collected and weighted. Shoots and roots were dried for 2 days at 75°C to obtain dry weights. Seed yield was calculated by multiplying the number of pod per plant by the number of seeds per pod and the seed weight mean. After drying, shoots were grinded and digested according to the EN 13805:2014. A segmented flow analyzer was used for total N evaluation (Skalar Inc. SanPlus, The Netherlands) and inductively coupled plasma optical emission spectrometry (ICP-OES; GBC Quantima, Australia) for total P and K. The ICP-OES operating conditions were as follows: 1000 W RF power, 15.0 L min^{-1} plasma gas flow rate, 1.2 L min^{-1} auxiliary gas flow rate, 1.0 L min^{-1} carrier gas flow rate, 50 scan/reading, 3 measurement replicates and dual detector.

Mycorrhizal Development

Mycorrhizal colonization in the roots of cowpea was assessed by microscopic methods. The roots were carefully washed and stained as described in a modified Phillips and Hayman (1970) protocol (Oliveira et al., 2005). The percentage of RLC was evaluated by the grid-line intersect method (Giovannetti and Mosse, 1980) under a stereomicroscope (Leica EZ4 HD, Germany).

Statistical Analysis

Normality and homogeneity of variances were confirmed and data analyzed by one-way and two-way analysis of variance (ANOVA) for each dependent variable versus the independent variables (inoculation and water regime). In some cases, transformation was performed before analysis, to normalize skewed distributions before ANOVA. This was the case of data of mycorrhizal colonization (x2), N shoot concentration (1/x), stomatal conductance (x1/3), transpiration rate ($\sqrt{x}$), water use efficiency (1/x) and carotenoids leaf content ($\sqrt{x}$). The main effects of the factors inoculation (Control, PPcoat, RIcoat and MIXcoat), water regime (D0, D1 and D2) and their interaction were analyzed. When a significant F-value was obtained ($P < 0.05$), treatment means were compared using Duncan's multiple range test. All statistical analyses were performed with the SPSS 25.0.0 software package (IBM SPSS Statistics, USA).

Results

Plant Growth, Yield and Nutrients Concentration

Seeds coated with *R. irregularis* inoculum (singly or mix) took approximately 7 days to final emergence from the soil, while those inoculated with bacteria and control took 4 days. Shoots, roots and total dry weights of cowpea were negatively affected by water regime, especially by severe water deficit (Tables 3.1 and 3.2). In general, the roots and total biomass were significantly affected by the inoculation treatments, positively by *P. putida* and negatively by *R. irregularis* (Table 3.2). There was no significant effect of inoculation on SDW under the different water regimes when compared with control (Table 3.1). Overall, PPcoat treatment had a significant enhancement effect in total plant dry weight, seed weight and seed yield of cowpea (Table 3.2). Under moderate water deficit, plants inoculated with *P. putida* presented a significant increase in seed yield (Table 3.1). RIcoat treatments presented lower root biomass when compared with the PPcoat and control treatments and consequently inferior values of root biomass over shoot (Table 3.2). The seed yield was significantly impaired by the severe water deficit (Table 3.2). Inoculation and water regime had significant main effects on cowpea shoot nutrients concentration (Table 3.2). In general, the presence of *R. irregularis* increased N and P shoot concentrations when compared

with control (Table 3.2). Yet, the interaction between water regime and inoculation showed only significant increase of N in plants under no water deficit (Figure 3.1), with an increase of 38% in shoot concentration. Comparing with the corresponding control, P shoot concentration was significantly increased in the treatments of RIcoat D0, Mix D1 and D2 by 39%, 85% and 57%, respectively. The accumulation of K in cowpea shoots was mainly affected by the water regime, being increased by moderate and severe water deficits (Table 3.2). Singly inoculated *P. putida* improved K shoot concentration by 25% under moderate water deficit (Figure 3.1).

Table 3.1. Biomass production and seed yield of *cowpea* under different inoculation treatments [non-inoculated (Control) *Rhizophagus irregularis* (RIcoat), *Pseudomonas putida* (PPcoat) and the mix *R. irregularis* + *P. putida* (MIXcoat)] and no water deficit (D0), moderate water deficit (D1) and severe water deficit (D2).

Inoculation	Water regime	SDW (g)	Root dry Weight (g)	Total plant dry weight (g)	Root/Shoot ratio	Seed weight (g)	Seed yield (g)
Control	D0	1.2±0.1 cd	0.9±0.1 e	2.0±0.2 g	0.8±0.0 abc	0.07±0.1 ab	0.4±0.1 a
	D1	0.7±0.0 b	0.5±0.1 cd	1.2±0.1 def	0.7±0.1 ab	0.10±0.0 ab	0.5±0.1 a
	D2	0.4±0.0 a	0.3±0.1 abc	0.8±0.1 bc	0.8±0.1 bc	0.08±0.0 ab	0.2±0.1 a
PPcoat	D0	1.3±0.1 d	0.9±0.1 e	2.1±0.1 g	0.7±0.0 ab	0.22±0.0 c	1.0±0.2 c
	D1	0.8±0.0 b	0.6±0.1 d	1.4±0.1 ef	0.8±0.1 bc	0.15±0.0 bc	0.9±0.0 bc
	D2	0.4±0.0 a	0.5±0.1 cd	0.9±0.1 bcd	1.1±0.2 c	0.12±0.0 abc	0.4±0.1 a
RIcoat	D0	1.1±0.1 c	0.4±0.0 bcd	1.5±0.1 f	0.4±0.0 a	0.06±0.0 a	0.3±0.2 a
	D1	0.6±0.1 b	0.2±0.0 ab	0.8±0.1 bc	0.4±0.1 ab	0.08±0.0 ab	0.4±0.1 a
	D2	0.3±0.1 a	0.1±0.0 a	0.4±0.1 a	0.6±0.2 ab	0.07±0.0 ab	0.2±0.1 a
MIXcoat	D0	1.0±0.0 c	0.4±0.1 bcd	1.4±0.1 ef	0.4±0.1 a	0.05±0.0 a	0.4±0.1 a
	D1	0.8±0.1 b	0.3±0.1 abc	1.1±0.1 cde	0.5±0.1 ab	0.11±0.0 ab	0.5±0.1 ab
	D2	0.4±0.1 a	0.3±0.1 abc	0.7±0.2 ab	0.6±0.2 ab	0.2±0.0 a	0.2±0.1 a

Means (± 1 SE) followed by letters that indicate significant differences between treatments according to Duncan's multiple range test at $P < 0.05$. SDW, shoot dry weight.

Table 3.2. Main effects of the factors inoculation and water regime and two-way ANOVA *F*-values and significances for biomass production, seed yield and nutrient shoot concentration of cowpea.

Main Effects		SDW (g)	Root dry Weight (g)	Total plant dry weight (g)	Root/Shoot ratio	Seed weight (g)	Seed yield (g)	N (g kg^{-1})	P (g kg^{-1})	K (g kg^{-1})
Inoculation (I)	Control	0.7 ab	0.6 b	1.3 b	0.8 b	0.1 a	0.4 a	12.3 b	1.5 a	25.3 a
	PPcoat	0.8 b	0.6 b	1.5 c	0.9 b	0.2 b	0.7 b	9.3 a	1.4 a	27.3 a
	RIcoat	0.7 a	0.3 a	0.9 a	0.5 a	0.1 a	0.3 a	14.7 c	2.0 b	26.1 a
	MIXcoat	0.7 a	0.3 a	1.0 a	0.5 a	0.1 a	0.4 a	15.4 c	2.3 c	26.4 a
Water regime (WR)	D0	1.1 z	0.6 z	1.7 z	0.6 x	0.1 x	0.5 y	12.2 x	1.8 x	22.6 x
	D1	0.7 y	0.4 y	1.2 y	0.6 x	0.1 x	0.6 y	12.5 x	1.6 x	27.0 y
	D2	0.4 x	0.3 x	0.7 x	0.8 y	0.1 x	0.2 x	13.1 x	1.8 x	30.6 z
Two-way ANOVA *F*-values and significances										
Inoculation (I)		3.8*	19.9***	16.0***	8.3***	8.0***	9.2***	17.9***	15.0***	3.3*
Water regime (WR)		140.5***	24.0***	85.5***	3.8*	1.4 ns	8.9***	0.9 ns	2.9 ns	41.0***
I x WR		1.2ns	1.5 ns	1.5 ns	0.5 ns	1.1 ns	1.1ns	0.9 ns	3.8**	1.0 ns

Letters indicate significant differences according to Duncan's Multiple Range test. *, **, ***, significant effect at the level of $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively; ns, non-significant effect. Control, non-inoculated; PPcoat, *Pseudomonas putida*; RIcoat, *Rhizophagus irregularis*; MIXcoat, mix of *R. irregularis* and *P. putida*; D0, no water deficit; D1, moderate water deficit; D2, severe water deficit. SDW, shoot dry weight.

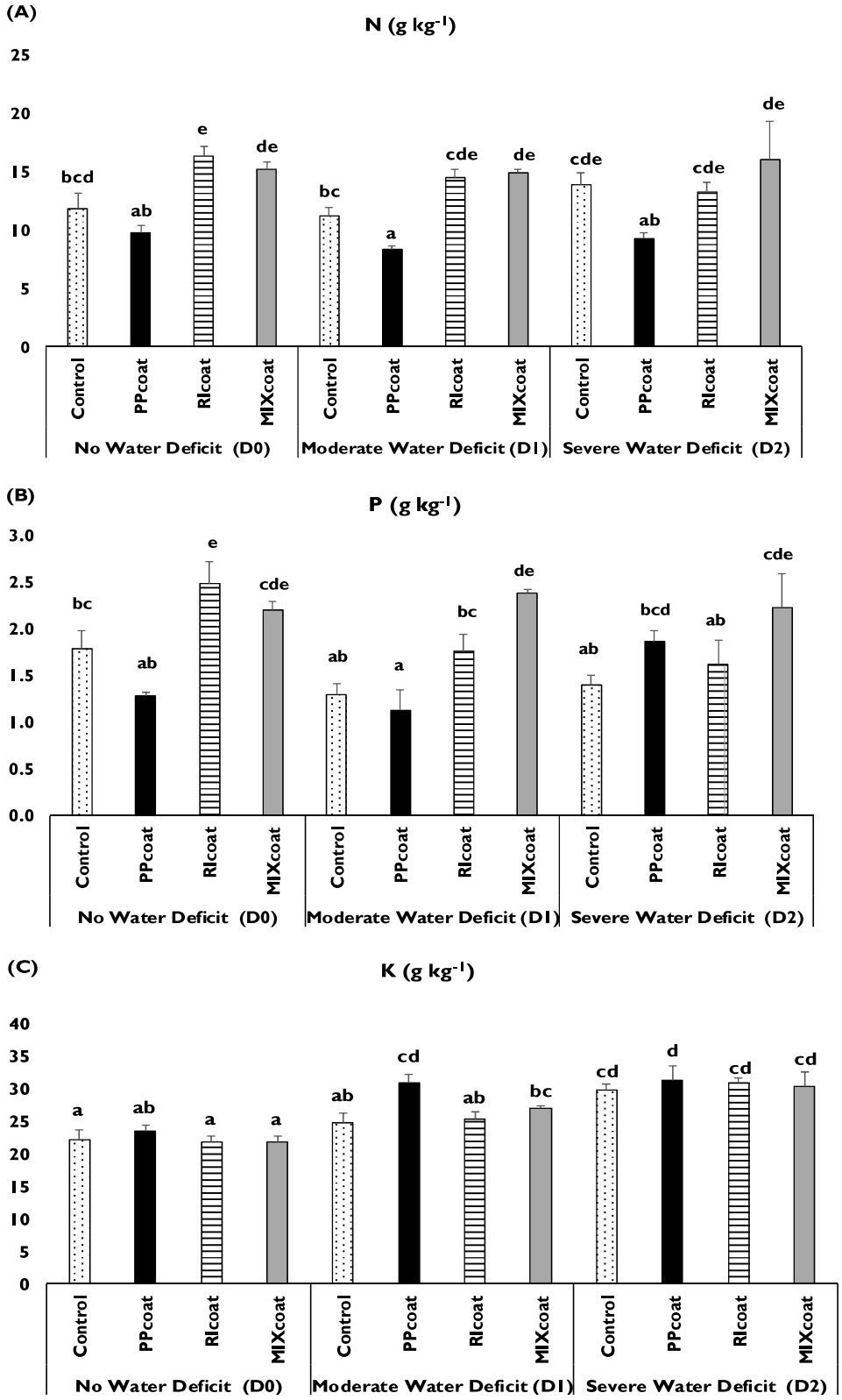


Figure 3.1. Effects of different inoculation treatments [non-inoculated (Control), with *Rhizophagus irregularis* (RIcoat), *Pseudomonas putida* (PPcoat) and the mix *R. irregularis* + *P. putida* (MIXcoat)] and water regimes on N (A), P (B) and K (C) shoot concentration in cowpea. Values are means ± 1 SE and letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

Mycorrhizal Root Colonization

Plants without *R. irregularis* inoculation (control and *P. putida* inoculation) had no root mycorrhizal colonization. Treatments where *R. irregularis* was inoculated had root colonization that varied with water regime (Figure 3.2). Both moderated and severe water restrictions negatively affected the presence of *R. irregularis* in the roots. When no water deficit was imposed, the percentage of RLC was higher than 50%. Inoculation with P. putida did not have a significant impact on root colonization by *R. irregularis*.

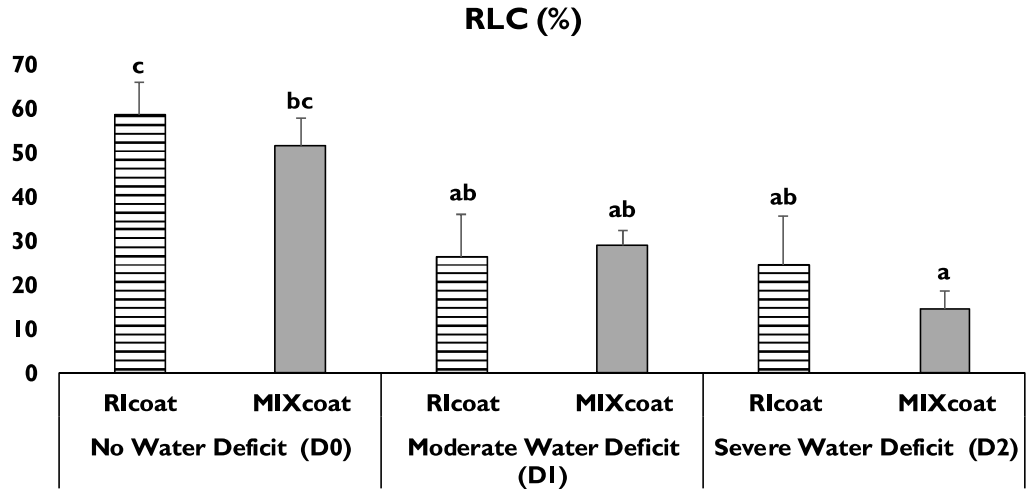
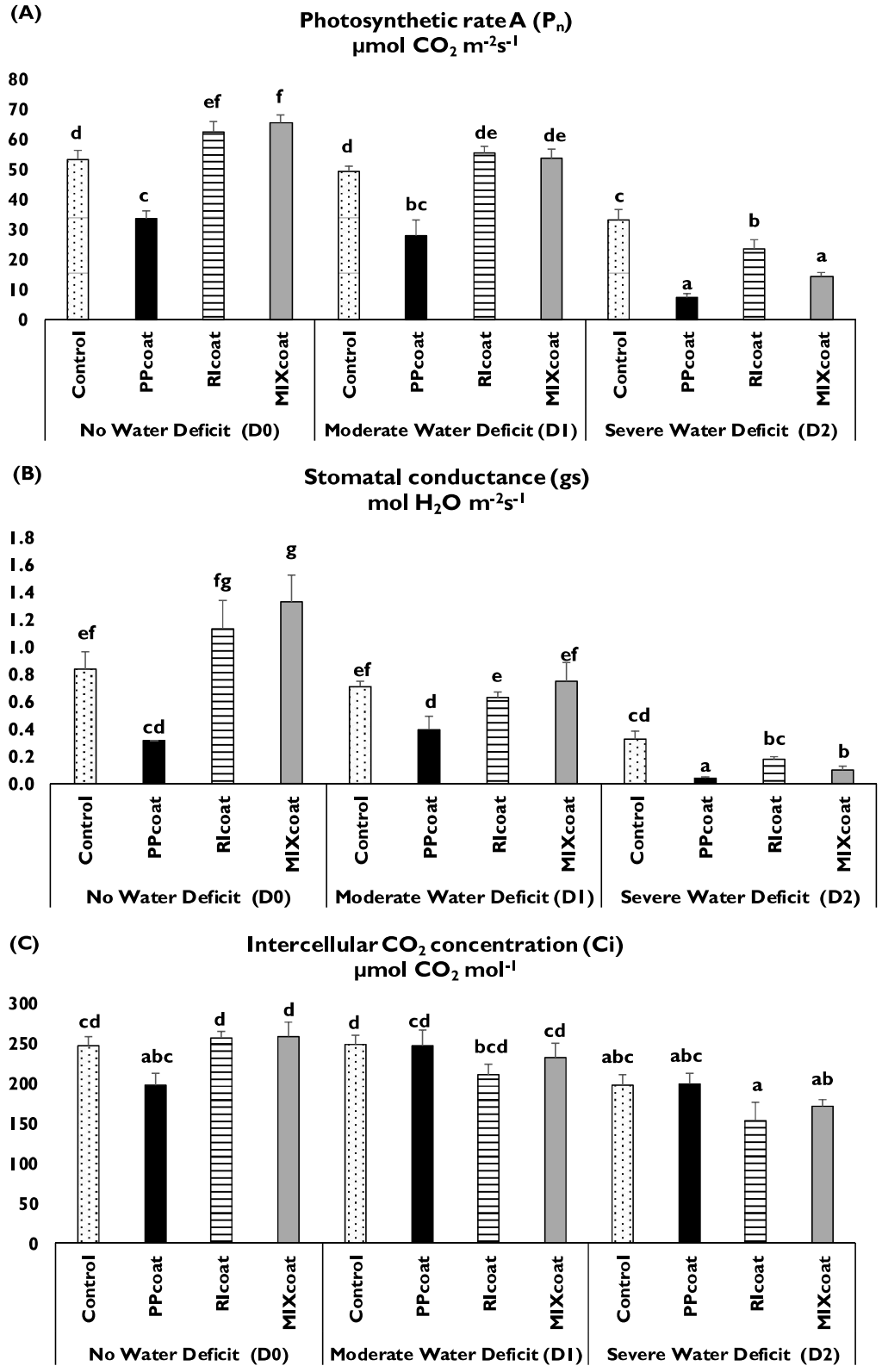


Figure 3.2. Percentage of root length colonization (% RLC) in the roots of cowpea inoculated with *Rhizophagus irregularis* (RIcoat) or the mix *R. irregularis* + *Pseudomonas Putida* (MIXcoat) via seed coating under different water regimes. Values are means ± 1 SE and letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

Leaf Parameters

Both water regime and microbial inoculation influenced cowpea leaf gas exchange parameters (Figure 3.3a–e and Table 3.3). Severe water deficit negatively affected the gas exchange parameters in both non-inoculated and inoculated treatments (Figure 3.3 and Table 3.3). The presence of mycorrhiza singly and in combination with *P. putida* significantly enhanced Pn when no water deficit was imposed (Figure 3.3a). Also, under no water deficit, the treatment MIXcoat presented higher values of gs and Tr (Figure 3.3b, d). Intercellular CO_2 concentration was adversely impacted by severe water deficit (Table 3.3). Plants singly inoculated with *P. putida* showed the lower values of Pn, gs and Tr in all water regimes. WUE (Figure 3.3e) was significantly higher in plants under severe water deficit and in the presence of microbial inoculants.

Chlorophyll and carotenoids varied according to microbial inoculation and water regime (Figure 3.4 and Table 3.3). Plants under moderate and severe water deficit had significantly lower concentrations the leaf pigments, irrespective of microbial inoculation (Table 3.3). In general, plants inoculated with *R. irregularis* enhanced both chlorophylls and carotenoids contents, even under severe water deficit, when compared with PPcoat and control treatments (Table 3.3).


(A)
Photosynthetic rate A (P_n)
µmol CO_2 $m^{-2}s^{-1}$
80
70
60
50
40
30
20
10
0
d
c
ef
f
d
bc
de
de
c
a
b
a
Control
PPcoat
Rlcoat
MIXcoat
No Water Deficit (D0)
Moderate Water Deficit (D1)
Severe Water Deficit (D2)
(B)
Stomatal conductance (gs)
mol H_2O $m^{-2}s^{-1}$
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ef
cd
fg
g
ef
d
e
ef
cd
a
bc
b
(C)
Intercellular CO_2 concentration (Ci)
µmol CO_2 mol^{-1}
300
250
200
150
100
50
0
cd
abc
d
d
d
cd
bcd
cd
abc
abc
a
ab

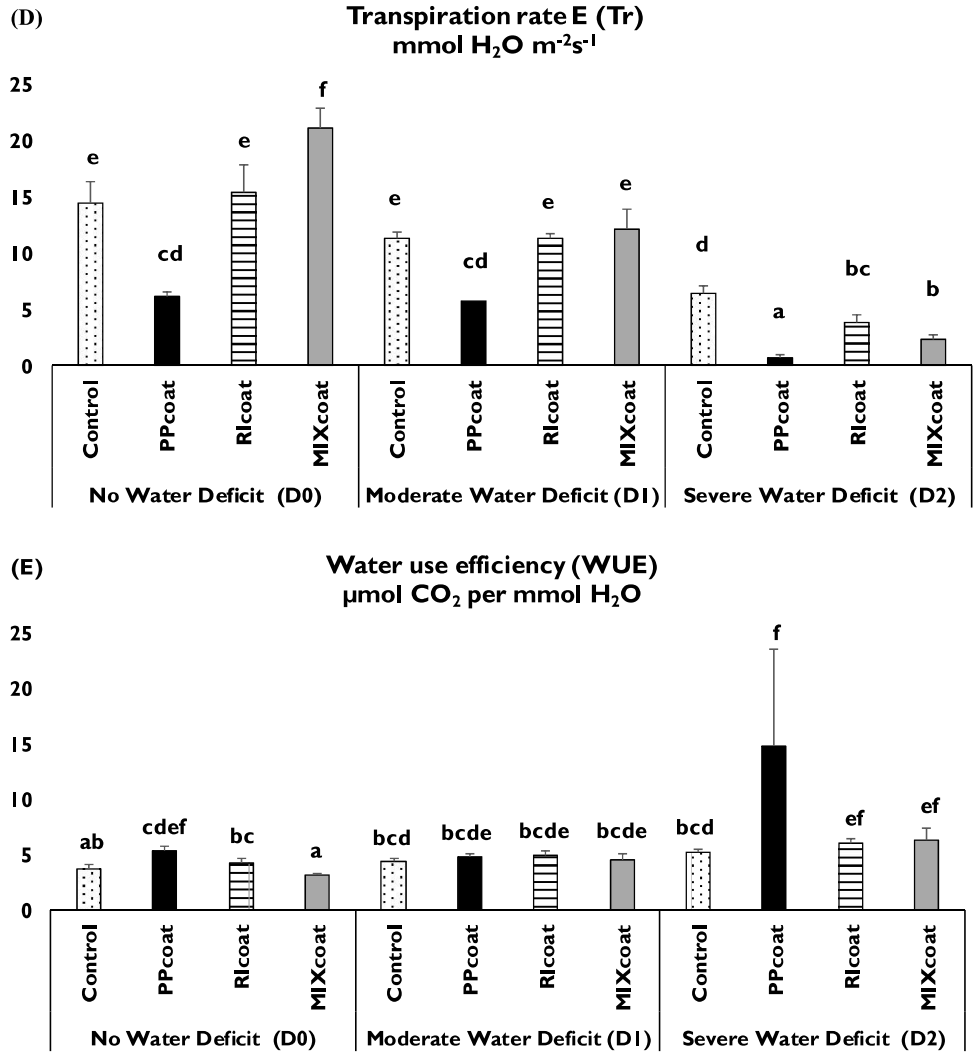


Figure 3.3. Effects of microbial inoculation [non-inoculated (control), *Rhizophagus irregularis* (RIcoat), *Pseudomonas putida* (PPcoat) and the mix of *R. irregularis* + *P. putida* (MIXcoat)] and water regime on Pn (A), gs (B), Ci (C), Tr, (D) and water use efficiency (WUE) (E) of cowpea. Letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

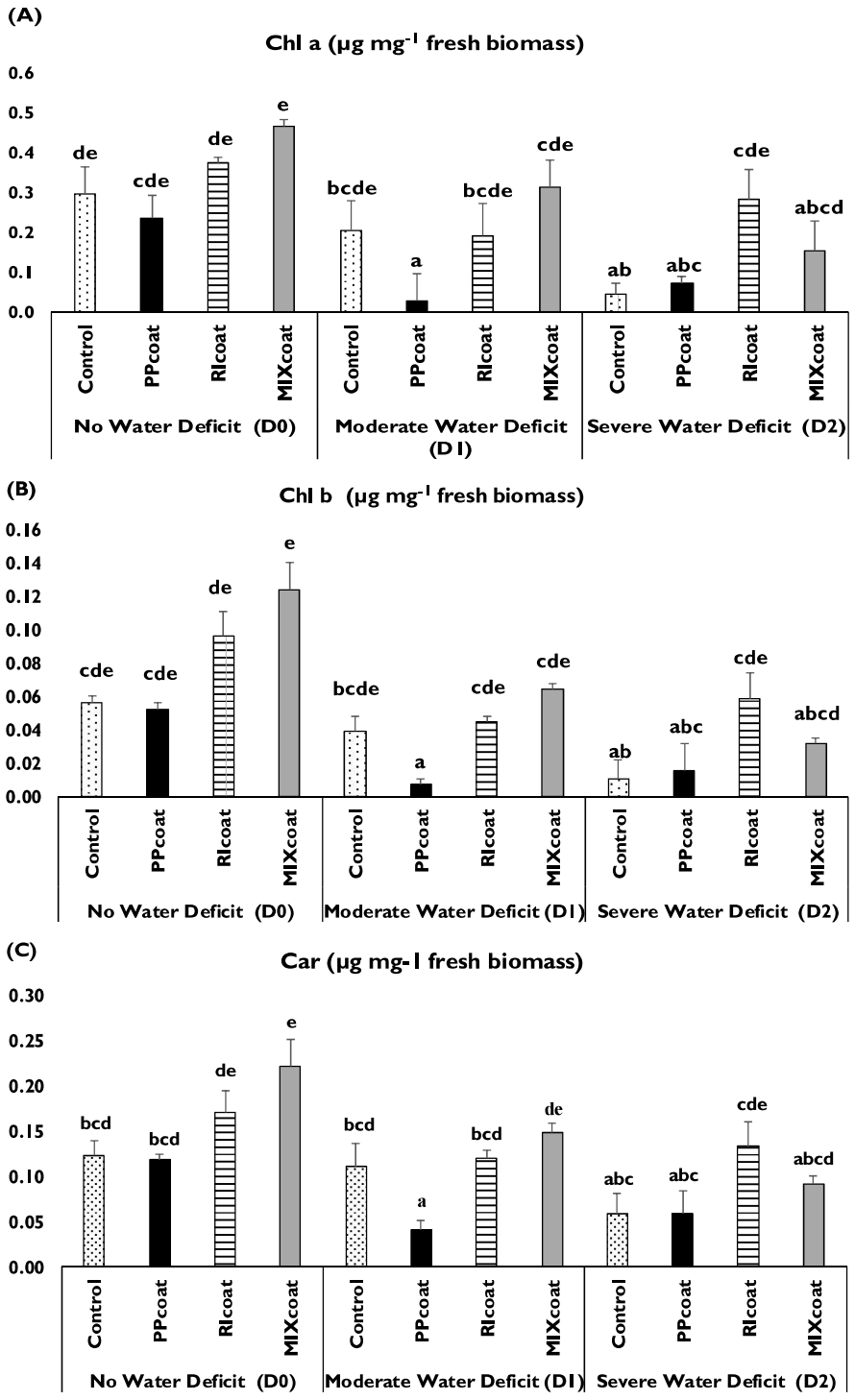


Figure 3.4. Chlorophyll a (a), chlorophyll b, (b) and carotenoids (c) leaf concentrations of cowpea under different inoculation treatments [non-inoculated control (control) and inoculated with *Rhizophagus irregularis* (Rlcoat), *Pseudomonas putida* (PPcoat) and the mix of *R. irregularis* + *P. putida* (MIXcoat)] and water regimes. Letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

Table 3.3. Main effects of the factors inoculation and water regime and two-way ANOVA *F*-values and significances for leaf parameters and chlorophyll and carotenoids contents of cowpea.

Main Effects		Photosynthetic rate A (P_n) µmol CO_2 $m^{-2}s^{-1}$	Stomatal conductance (gs) mol H_2O $m^{-2}s^{-1}$	Intercellular CO_2 concentration (Ci) µmol CO_2 mol^{-1}	Transpiration rate E (Tr) mmol H_2O $m^{-2}s^{-1}$	Water use efficiency (WUE) µmol CO_2 per mmol H_2O	Chlorophyll a (µg mg^{-1} FL)	Chlorophyll b (µg mg^{-1} FL)	Carotenoids (µg mg^{-1} FL)
Inoculation (I)	Control	46.40 b	0.67 b	233.20 a	11.11 b	4.40 a	0.18 ab	0.04 a	0.07 a
	PPcoat	22.87 a	0.25 a	215.00 a	4.26 a	8.34 c	0.11 a	0.02 a	0.10 a
	RIcoat	47.27 b	0.65 b	206.78 a	10.21 b	5.09 bc	0.28 b	0.07 b	0.14 b
	MIXcoat	46.60 b	0.78 b	224.80 a	12.83 b	4.54 ab	0.31 b	0.07 b	0.15 b
Water regime (WR)	D0	54.62 z	0.94 z	241.92 y	14.83 z	4.05 x	0.34 y	0.08 y	0.16 y
	D1	47.37 y	0.65 y	235.85 y	10.49 y	4.62 y	0.18 x	0.04 x	0.10 x
	D2	19.58 x	0.16 x	180.42 x	3.36 x	8.12 z	0.14 x	0.03 x	0.14 x
Two-way ANOVA *F*-values and significances									
Inoculation (I)		43.87***	22.13***	1.42 ns	32.70***	5.65**	4.62*	5.36**	6.91**
Water regime (WR)		145.14***	94.78***	18.92***	112.87***	18.34***	9.37***	10.50***	8.54**
I x WR		5.90***	3.75**	2.82*	4.61**	1.77 ns	1.36 ns	1.88 ns	1.15 ns

Letters indicate significant differences according to Duncan's Multiple Range test. *, **, ***, significant effect at the level of $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively; ns, non-significant effect. Control, non-inoculated; PPcoat, *Pseudomonas putida;* RIcoat, *Rhizophagus irregularis*; MIXcoat, mix of *R. irregularis* and *P. putida*; D0, no water deficit; D1, moderate water deficit; D2, severe water deficit. FL, stands for fresh leaf.

Discussion

The frequency and intensity of drought can dramatically decrease plant biomass and grain yield (Farooq et al., 2017). Ahmed and Suliman (2010) showed cowpea yield reductions of 34–66% under water stress during the reproductive stage of crop development, and Akyeampong (1986) revealed 29% of declination during pod filling. Our results showed that both moderate and severe water deficit decreased shoots, roots and total biomass and that severe water deficit significantly reduced seed yield (Table 3.2). The negative variation on gas exchange parameters such as photosynthesis, stomatal conductance or transpiration imposed by water stress can hamper plant growth (Farooq et al., 2008; Li et al., 2014), which was shown in our results (Table 3.1 and Figure 3.3). Equally, water deficit significantly decreased the content of chlorophyll a, chlorophyll b and carotenoids in cowpea leaves (Table 3.3). Photosynthetic pigments are important for plants to harvest light and produce reducing powers. Carotenoids play a key role in plant antioxidant defense system by quenching singlet oxygen and peroxyl radicals, protecting the photosynthetic tissue from oxidative damage (Jaleel et al., 2009).

Legume crops are able to establish symbiotic interactions with microbes (e.g. PGPR and AM fungi), which help them cope with unfavorable environmental conditions such as drought (Oliveira et al., 2017a; 2017b; Zahran, 2010). Cowpea is considered to be highly mycotrophic (Molla and Solaiman, 2009) which leads to enhancement of below and above ground biomass, nutrients accumulation, protein content and grain yield under different water regimes (Kwapata and Hall, 1985; Oliveira et al., 2017a; Oruru et al., 2018; Rabie et al., 2005). However, our results showed that association between AM fungi and cowpea did not result in increased plant growth or seed yield (Tables 3.1 and 3.2). Moreover, for root weight and root/shoot ratio the values of plants inoculated with *R. irregularis* were lower than control. This can be related to the fact that the production of fungal mycelium is much more cost-effective in terms of organic carbon (C) than the production of equivalent root length (Table 3.2). Consequently, plants adjust belowground C allocation contributing to the formation of a shorter mycorrhizal root system (Jacobsen et al., 2002), relying on the fungal mycelium for nutrient uptake (Smith, 2000). In fact, there was a significant enhancement in shoot nutrient content (Table 3.2), particularly N and P, which has also been described in other studies with inoculated cowpea (Boby et al., 2008; Oruru et al., 2018; Sanginga et al., 2000; Yaseen et al., 2011). Still, this enhancement in nutrient content was not enough to result in greater yields, fact perhaps associated with the sink of carbohydrates of the fungal mycelium that the plant could not allocate to seed development and filling. Also, the observed delay on seedling emergence of plants inoculated with AM fungi might have a negative influence on cowpea yield or even adaptation to the water deficit. Faster germination and establishment increases the opportunity of seedlings to achieve a positive C and nutrient balance, which is crucial,

especially under stress conditions (de Albuquerque and Carvalho, 2003). Further studies are, therefore, needed to improve this limitation on the germination of cowpea seeds coated with AM fungi. On the other hand, when compared with control, there was an overall enhancement on chlorophyll and carotenoids contents in *R. irregularis*-inoculated plants (Table 3.3), particularly under severe water deficit for chlorophyll a and b (Figure 3.4). WUE, one of the mechanisms of plants to increase drought resistance (Vivas et al., 2003), was increased in plants inoculated with *R. irregularis* and *P. putida* under severe water deficit (Figure 3.3). The presence of mycorrhiza significantly enhanced photosynthetic rate, stomatal conductance and transpiration rate (Figure 3.3) under no water deficit, corresponding to the water regime where the colonization was higher (Abdel-Salam et al., 2018). The increased rate of photosynthesis was probably a result of the increased use of fixed C (Fitter, 1991) and/or higher chlorophyll content (Gusain et al., 2015), under no water deficit (Figures 3.3 and 3.4). Under severe water deficit, this relationship between photosynthesis and chlorophyll content was not so obvious. Water deficit affects various physiological and biochemical processes of plants, limiting stomata and transpiration and resulting in reduced photosynthesis (Farooq et al., 2009). These physiological limitations and decreased photosynthetic rate under water deficit possibly eliminated the compensatory effect of mycorrhiza shown in plants without water deficit. In fact, under water deficit the decrease in photosynthetic activity was also greater in mycorrhizal plants, as shown by Birhane et al. (2012). Thus, this photosynthetic depression could have been responsible for the lower percentage of AM root colonization. AM fungal colonization is negatively influenced by water deficit (Kaya et al., 2003; Oliveira et al., 2017a; Wu and Xia, 2006), which, in the present study, might have been related to the observed reduction of cowpea fitness and to the lower production of photosynthates, meaningless C for the fungal symbiont.

PGPR singly or in combination with AM fungi play a significant role in alleviating drought stress in plants (Vurukonda et al., 2016). In our results, the co-inoculation (PGPR + AM fungi) apparently did not present any extra benefit to the plants. On the other hand, plants singly inoculated with *P. putida* showed a significant increase in seed yield (Table 3.2), including under moderate water deficit (Table 3.1). Overall, *P. putida* significantly enhanced total plant biomass (Table 3.2). The accumulation of K in cowpea shoots was enhanced by 25% in plants singly inoculated with *P. putida* under moderate water deficit (Figure 3.1). K is an essential nutrient for plants and plays an important role in drought conditions, cell membrane stability, root growth and leaf area increase, water uptake and water conservation improvement (Wang et al., 2013). The enhancement of K under moderate water deficit might be one of the factors responsible for improving cowpea tolerance to the stress and positively influencing seed yield, when comparing to the reaming treatments under the same water regime. The ability of PGPR to increase plant biomass, yield and protein content both under greenhouse and field conditions was shown before in legumes (Oliveira et al., 2017a; 2017b; Sindhu et al., 2010). Many studies with various crops showed a positive relationship between PGPR inoculation and drought tolerance (Figueiredo et al., 2008;

Gusain et al., 2015; Kohler et al., 2008; Naseem and Bano, 2014). In these studies, the production of phytohormones and the production of exopolysaccharides helped with drought stress alleviation and/or increased seed yield and protein content. The increase of drought stress tolerance by PGPR can be related to several mechanisms, such as production of phytohormones (abscisic acid, gibberellic acid, cytokinins and IAA); ACC deaminase; inducer of SAR; and production of exopolysaccharides (Vurukonda et al., 2016). The *P. putida* strain used in the present study is a strong IAA producer, which is physiologically the most active auxin in plant growth and development. More studies on the microbial mechanisms behind the increase in drought stress tolerance and yield are essential.

Conclusions

It is imperative to improve agricultural productivity, in a sustainable way, against unfavorable environmental conditions. Understanding plant responses to drought is of great importance, since this is one of the main constraints to crop yield. Microbial inoculation is known to confer drought resistance to plants. In this study, results showed a general positive effect of bacterial inoculation via seed coating on crop productivity under moderated water deficit, which might be relevant for agricultural applications. AM fungal inoculation via seed coating had an overall positive influence on cowpea regarding the uptake of nutrients, leaf pigments content and gas exchange parameters, nonetheless mostly obtained under no water deficit. The application of PGPR and AM fungi represents a key approach for agricultural systems and should be integrated with or without drought stress, yet more studies concerning the microbe–plant interaction and the mechanisms that confer the stress alleviating abilities are necessary. Selecting the microbe that better potentiates plant tolerance is critical for the efficiency of microbial inoculation. On the other hand, seed coating can be a promising tool for efficiently delivering microbial inocula. Nonetheless, additional studies are needed to address the cowpea seed germination reduction and improve the technique. Moreover, field studies under real agricultural context are indispensable to prove the possible application of seed coating with PGPR and AM fungi in a large-scale approach.

Chapter 4 - Seed coating with arbuscular mycorrhizal fungi for improved field production of chickpea

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Abstract

Although arbuscular mycorrhizal (AM) fungi are known to promote growth and yield of agricultural crops, inoculation methods for effective scaling up from greenhouse to the field are still underexplored. The application of single or mixed beneficial AM fungal isolates is hindered by the lack of experimental reproducibility of findings at different scales and the cost-effectivity of inoculation methods. Seed coating has been considered a feasible delivery system of AM fungal inocula for extensive agricultural crops. In this study, the impact of single and multiple AM fungal isolates applied via seed coating on chickpea productivity was evaluated under greenhouse and field conditions. Overall, plants inoculated with multiple AM fungal isolates had better performance than those inoculated with single AM isolate under greenhouse and field conditions. While plants in greenhouse displayed higher SDW (14%) and seed individual weight (21%), in field, inoculation with multiple AM isolates increased pod (160%), and seed (148%) numbers, and grain yield (140%). Under field conditions, mycorrhizal root colonization was significantly higher in chickpea plants inoculated with multiple AM fungal isolates compared to other treatments. These findings highlight the potential of field-inoculation with multiple AM fungal isolates via seed coating as a sustainable agricultural practice for chickpea production.

Keywords: Arbuscular mycorrhizal fungi; *Cicer arietinum* L.; Field crop production; Seed coating.

Introduction

Chickpea (*Cicer arietinum* L.), one of the main legume crops consumed and cultivated worldwide (Iqbal et al., 2006; FAOSTAT, 2017), is considered an important and cheap source of nutrients (Jukanti et al., 2012) as well as a key crop for soil fertility preservation, especially in rainfed areas (Khan et al., 2011). Arbuscular mycorrhizal (AM) fungi are known to promote the growth and yield of legumes, including chickpea (Farzaneh et al., 2011; Pellegrino and Bedini, 2014; Oliveira et al., 2017b; Hashem et al., 2018). According to Rillig (in press), the integration of AM fungi in agricultural management strategies is recommended not only for their contribution to crop yield increase, but also for the important roles in ecosystem functions (e.g. soil structure, nutrient conservation, plant stability over changing environment) and potential to reduce the amounts of fertilizer required to achieve cost-effectiveness. AM fungi are capable of increasing the efficiency of agricultural systems through different mechanism such as nutrient uptake regulation, water balances and plant resistance to biotic stresses (Kempel et al., 2010; Hameed et al., 2014; Kumar et al., 2016; Oliveira et al., 2016a; Singh et al., 2016; Frew et al., 2017). Additionally, AM

fungi can also have a positive and significant influence over grain/seed quality (Ryan and Graham, 2018; Rillig, 2019). However, factors such as host plant affinities, soil conditions and the use of single versus multiple AM fungal isolates can have a great impact on the performance of these beneficial microbes (Van Der Heijden et al., 2015; Kim et al., 2017). According to Frew (2019), agricultural crops can, in general, benefit from higher AM fungal diversity (multiple isolates) in the soil, yet the growth and nutritional advantages depend on the plant-host species. On the other hand, the application of AM fungi in agricultural systems is still restricted due to the lack of cost-effective inoculation methods or the reproducibility of results from greenhouse and field tests (Vosátka et al., 2012; Malusà et al., 2016; O'Callaghan, 2016). In this sense, strategies for developing microbial inoculation methods for broad-scale agricultural production that effectively apply low amounts of inoculants are required.

Seed coating is a process consisting on the application of exogenous materials (including inoculants) onto the seed surface and it has been considered a precise tool with the potential to deliver AM fungi to several agricultural crops, such as wheat, maize, artichoke and cowpea (Oliveira et al., 2016b; Rouphael et al., 2017; Rocha et al., 2019; Rocha et al., in press; Ma et al., 2019). Seed coating ensures the contact of AM fungal propagules with emerging roots assuring colonization at the early plant development stage. Regardless of the potential to increase the productivity and nutrition of different agricultural crops (Cely et al., 2016; Oliveira et al., 2016b, Rocha et al., 2019), inoculation of AM fungi via seed coating is still scarce. The scaling up from laboratory tests, through greenhouse studies and finally to field conditions is a challenging task in the selection or elaboration of effective mixtures and inoculation methods to apply beneficial microbes (Lugtenberg and Kamilova, 2009). Studies that contemplate more than one experimental scale are still scarce and it is crucial to understand the biases of inoculation performance, since the beneficial effects of microbial inoculation obtained under greenhouse conditions are not always achieved in the field (Hart et al., 2018). To our knowledge, no field studies focusing on seed coating inoculation of chickpea with AM fungi have been reported so far.

In our study it is expected that inoculation of multiple AM fungal isolates results in superior chickpea performance when compared to single AM fungal isolate under both greenhouse and field conditions. Therefore, the objectives of this work were 1) to compare the efficiency of inoculation of single and multiple AM fungal isolates via seed coating and their effects on chickpea yield and nutritional content under greenhouse and field conditions, and 2) to verify whether results from AM fungal inoculation obtained under greenhouse conditions can serve as an indicator of their potential benefits for field applications.

Materials and Methods

Arbuscular Mycorrhizal Fungal Inocula and Seed Coating

Two different AM fungal inocula (provided by Symbiom Ltd, Czech Republic) were used, one consisted of a single fungal isolate *R. irregularis* BEG140 and the other was a mixture of equal proportions of five *R. irregularis* isolates namely BEG141, BEG236, DAOM 197198, KW and AS. Both fungal inocula were grown for 8 months in a multispore pot culture containing a 1:1 (v/v) mixture of zeolite and expanded clay with *Zea mays* L. as the host plant.

For the seed coating treatment, seeds were dusted with *R. irregularis* inoculum (sieved through 500 µm mesh) followed by biochar (0.25% of seed weight) (Ecochar, Ibero Massa Florestal, Portugal). Gum arabic solution (2%) was used as a binding agent. Chickpea seeds were dressed using a rotating pan according to Scott et al. (1991). Twenty AM fungal propagules were applied per seed and estimated by the MPN (Porter, 1979).

Experimental Design

The experimental design involved three treatments, resulting from three different inoculations including 1) non-coated and non-inoculated controls (control), 2) plants coated with *R. irregularis* BEG140 (Rcoat), and 3) a mixture of *R. irregularis* isolates (MRcoat). The effect of seed coating with different fungal combinations was evaluated under both greenhouse and field conditions. Both experiments were conducted simultaneously from April to August 2018. The seeds of chickpea (*Cicer arietinum* L. cv. Elixir) were obtained from the collection of the INIAV.

The field experiment was conducted at the INIAV station in Elvas, Portugal (38°55'07.8" North, 7°05'33.2" West, 209 meters above sea level). The field had been used for chickpea and oat production in a crop rotation system. The temperature fluctuated from 6 to 40 °C (average 13 to 26 °C), 42 to 78% of RH and 0 to 8 mm of precipitation. The soil had a clay texture with pH (1:2.5 w/v water) 7.5, electrical conductivity 0.30 mS cm^{-1}, 2.1% organic matter, 168 mg kg^{-1} extractable (Egner-Riehm) P, >200 mg kg^{-1} extractable (Egner-Riehm) K, 7174 mg kg^{-1} extractable (ammonium acetate) Ca and 206 mg kg^{-1} extractable (ammonium acetate) Mg. Eight months before starting the field experiment, the soil was amended with 200 Kg ha^{-1} of fertilizer with 20% N; 8% P and 10% K (NERGETIC C-PRO 20-8-10®, ADP Fertilizantes, Portugal). Each experimental plot consisted of three rows of 4 meters (with 30 seeds each and 60 cm between rows) that was organized in a split-plot randomized block with three repetitions per treatment. After the seed coating treatment, seeds were sown manually at 2 cm depth and separated by at least by 13cm. During the experiment, plants were grown under natural rainfall conditions without receiving further irrigation or fertilization.

For the greenhouse experiment, soil was collected from the same field, sieved (2 mm) and used in order to provide a similar soil microbiota and chemical properties. Ten replicates per treatment were disposed in individual plastic pots of 3 L (14 x 14 x 23 cm) that received one seed and were arranged in a fully randomized scheme. The pot positions were periodically swapped in order to minimize specific differences related to microsite location in the greenhouse. During the experiment, greenhouse temperature ranged from 14 to 42 °C (average 16 to 30 °C) and RH was maintained between 40 to 85%, with an average photoperiod of 12 h. In order to maintain soil humidity, pots were irrigated as frequently as required to restore water losses produced by evapotranspiration, on average 3 times a week.

Plant Measurements

In both field and greenhouse experiments, plants were harvested approximately 120 days after sowing (DAS). DAS required for germination, flowering (flowering of 50% of the plants) and maturity (maturity of 50% of the plants) were recorded. Pods and seeds were collected, counted and weighted to quantify grain yield per plant. Shoot samples from both experiments were dried at 70 °C for 48 h and weighed. For plants grown under field conditions, the weight of 100 seeds, harvest index of chickpea [Grain dry weight (GDW) / SDW] and the relative effectiveness (RE) of inoculation (SDW of inoculated plants / SDW non inoculated plants) were also calculated according to Maâtallah et al. (2002)

Crude Protein and Fiber, Fat and Ash Grain Content Analyses

After collection, grain samples were dried at 70 °C for 48 h and finely ground. The protein content was analyzed according to the Kjeldahl method [international standard (ISO) 20483:2006]. Crude protein was calculated by multiplying the N content by 6.25. The crude fiber content was quantified using the method with intermediate filtration from the Portuguese Norm (NP) EN ISO 6865:2009. After acid and alkaline digestion of the sample, the crude fiber content was calculated from the loss in mass resulting from ashing of the dried residue divided by the mass of the test portion. Finally, the grain fat content was determined with ether ethylic using the extraction apparatus Soxtec System HT1043 in accordance to the NP 876:2001. The ash yield was determined by incineration and calculated as a fraction of the mass of ashing dish and incinerated residue, divided by the mass of the test portion, according to the ISO 2171:2007.

Mycorrhizal Development

After harvest, roots from plants collected from greenhouse and field experiments were separated from shoots, gently washed tap water, cut into 1-cm pieces and stained with trypan blue using a modified Phillips and Hayman (1970) protocol (Oliveira et al., 2005). The percentage of RLC was assessed by the grid-line intersect method (Giovannetti and Mosse, 1980) under a stereomicroscope (Leica EZ4 HD, Germany).

Statistical Analysis

Normality and homogeneity of variances were confirmed and data analyzed using one-way analysis of variance (ANOVA). In the case of pod number and the number of pods with 2 grains, square (x^2) and root ($\sqrt{x}$) transformations were required to satisfy normality assumptions before ANOVA When a significant *F*-value was obtained ($P < 0.05$), treatment means were compared using Duncan's multiple range test. When normality assumptions were not met (as in the case of SDW, the weight of individual grains and ash content for field data and grain number and protein content for greenhouse data), differences between groups were compared using non-parametric Kruskal–Wallis test. SPSS 25.0.0 software package (IBM SPSS Statistics, USA) was used to perform all the statistical analyses.

Results

Growth Parameters

In general, chickpea seeds took approximately double time to germinate in the field (15 DAS) compared to the germination observed in the greenhouse (7 DAS). Nevertheless, seed coating with AM fungi did not affect germination rates of chickpea. Flowering and maturation times of cowpea plants were similar under greenhouse and field conditions; in the greenhouse, flowering and maturation took 46 and 101 DAS, whereas 42 and 102 DAS were necessary under field conditions, with no significant differences between inoculation treatments.

Under greenhouse conditions, single inoculation of *R. irregularis* BEG140 (Rcoat) did not show clear effects on chickpea plants when compared to control, with the exception of the grain individual weight. However, seed coating with the mixture of *R. irregularis* isolates (MRcoat) showed positive effects on chickpea productivity when compared with control treatment at both experimental scales (Table 4.1). Under greenhouse conditions, plants treated with the MRcoat treatment exhibited a significant increase in SDW (14%, $P < 0.001$) and also in the grain individual weight per plant (21%, $P < 0.05$). In the field, the effect of the coating treatment containing the *R. irregularis* consortia was much more noticeable. Here, MRcoat treatment produced a significant increase in valuable agronomic parameters as the number of pods per plant, seeds per pod and seeds per plant in comparison with the remaining treatments. Inoculation significantly enhanced the number of pods and grains by 160% ($P < 0.001$) and 148% ($P < 0.001$), respectively.

Table 4.1. Growth and productivity parameters of chickpea in different inoculation treatments [control, *Rhizophagus irregularis* (Rcoat), a mixture of *R. irregularis* isolates (MRcoat) under greenhouse and field conditions.

Experimental scale	Treatment	SDW (g)	Number of pods per plant	Number of pods with 2 grains per plant	Number of grains per plant	Weight of individual grains per plant (g)	Grain yield per plant (g)
Greenhouse	Control	1.4±0.1 a	8±0.6	NA	5±0.2	0.29±0.0 a	1.5±0.0
	Rcoat	1.3±0.0 a	7±0.1		5±0.4	0.33±0.0 b	1.5±0.1
	MRcoat	1.6±0.1 b	8±0.9		4±0.3	0.35±0.0 b	1.5±0.1
Field	Control	10.2±2.1	24±4.1 x	4±0.6 x	27±3.8 x	0.30±0.0	8.4±1.4 x
	Rcoat	6.0±1.1	25±7.6 x	3±1.4 x	25±7.9 x	0.33±0.0	7.9±2.5 x
	MRcoat	8.9±0.9	62±1.6 y	9±1.9 y	68±5.9 y	0.30±0.0	20.1±1.4 y

Means (± 1 SE) followed by letters that indicate significant differences between treatments within the same experimental scales according to Duncan's multiple range and Kruskal-Wallis test at $P < 0.05$. SDW, shoot dry weight. NA, not applied.

The grain yield of chickpea was not affected by AM fungal inoculation when grown under greenhouse (Table 4.1). On the other hand, MRcoat treatment significantly increased grain yield per plant (140%, $P < 0.001$) under field conditions. Consequently, harvest index was also significantly higher in the MRcoat treatment (Table 4.2). There were no significant differences between treatments in the weight of 100 seeds (32 g for all treatments).

Table 4.2. Harvest index (ratio of grain dry weight to shoot dry weight) of chickpea and relative effectiveness of inoculation (ratio of shoot dry weight of inoculated plant to shoot dry weight of non-inoculated plants) under field conditions.

Treatment	Harvest index (%)	Relative effectiveness (%)
Control	103.7 x	-
Rcoat	147.4 xy	66.0
MRcoat	229.8 y	107.0

Means followed by letters that indicate significant differences between treatments according to Duncan's multiple range test at $P < 0.05$. Rcoat (*Rhizophagus irregularis*), MRcoat (mixture of *R. irregularis* isolates).

A summary of the chickpea productivity parameters among the different treatments under greenhouse and field conditions is presented in Figure 4.1.

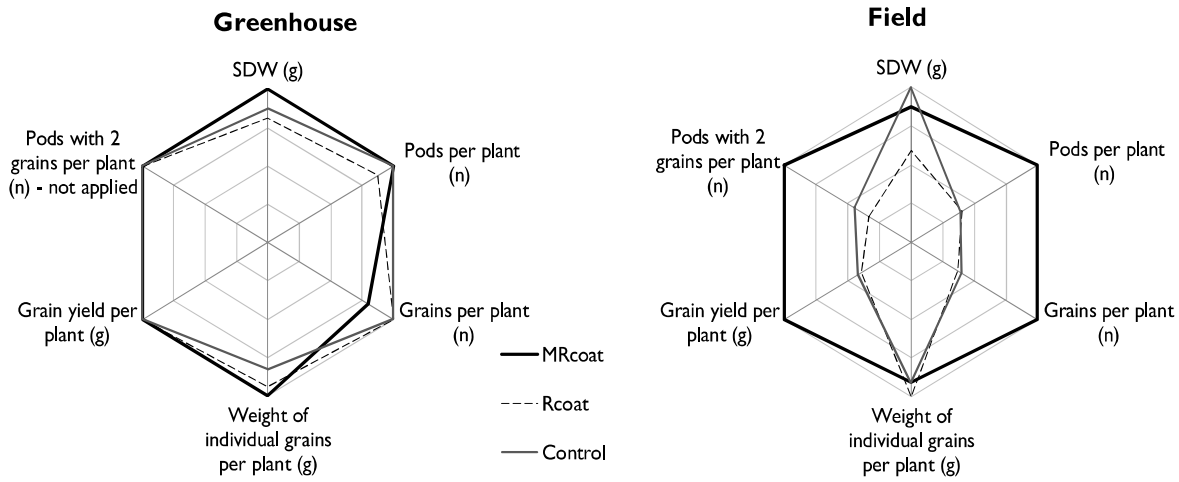

Figure 4.1. Representation of productivity parameters of chickpea in different inoculation treatments [control, *Rhizophagus irregularis* (Rcoat), a mixture of *R. irregularis* isolates (MRcoat)], under greenhouse and field conditions. Radial graphs represent results relative to the higher value (indicated as 100%) for each productivity parameter.

Grain Quality

In terms of nutritional quality, no significant differences in crude protein, fat, crude fiber and ash content of chickpea grains were detected in the greenhouse trial (Table 4.3). Under field conditions, no significant differences in protein, fat and ash grain content were detected among treatments with the exception of crude fiber that was significantly higher in non-inoculated plants.

Table 4.3. Chickpea grain content in different inoculation treatments [control, *Rhizophagus irregularis* (Rcoat), mixture of *R. irregularis* isolates (MRcoat) under greenhouse and field conditions.

Experimental Scale	Treatment	Crude protein (%)	Fat (%)	Crude fiber (%)	Ash (%)
Greenhouse	Control	19.9±0.4	4.2±0.1	4.5±0.4	2.5 ±0.1
	Rcoat	19.3±0.3	4.6±0.3	4.4±0.2	2.4±0.1
	MRcoat	18.4±0.5	4.5±0.2	4.6±0.6	2.3±0.1
Field	Control	20.8±0.5	3.9±0.1	4.1±0.2 y	3.0±0.2
	Rcoat	20.8±0.2	3.9±0.2	3.5±0.1 x	2.9±0.0
	MRcoat	20.6±0.5	3.9±0.3	3.1±0.1 x	2.6±0.0

Means (± 1 SE) followed by letters that indicate significant differences between treatments according to Duncan's multiple range test and Kruskal-Wallis test at $P < 0.05$.

Mycorrhizal Colonization

The percentage of RLC of plants grown in the greenhouse was not statistically different among treatments, with values of 65, 66 and 74% for control, Rcoat and MRcoat, respectively

(Figure 4.2). Under field conditions, MRcoat treatment showed higher rates of fungal colonization in their roots, 69% of RLC in comparison with 54 and 42% in control and Rcoat treatments, respectively.

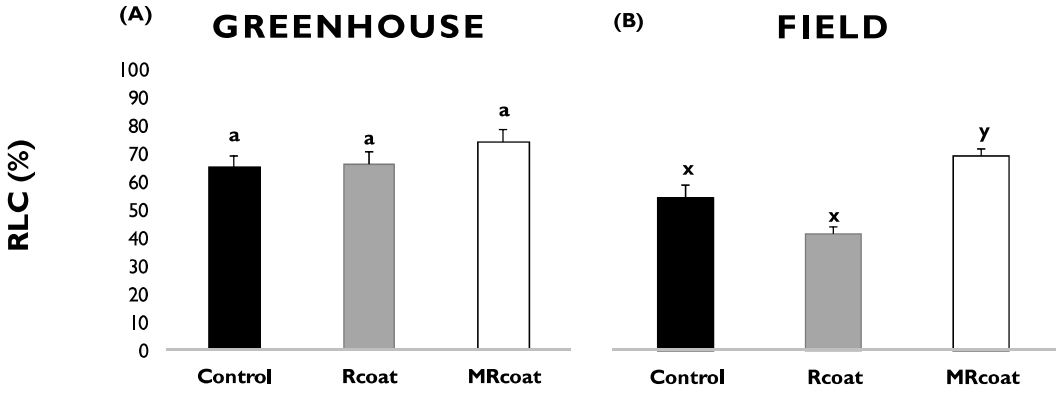

Figure 4.2. Percentage of root length colonization (% RLC) of chickpea non-inoculated (control) and inoculated with *Rhizophagus irregularis* BEG140 (Rcoat) or mixture of *R. irregularis* isolates (MRcoat) via seed coating in greenhouse (A) and field (B) trials. Columns are means ± 1 SE and letters indicate significant according to Duncan's Multiple Range test at $P < 0.05$.

Discussion

Difficulties to replicate in the field the effects observed under controlled greenhouse conditions are generally considered a major constrain to expand the use of beneficial microbes in agriculture. In order to address this pitfall, experimental conditions such as time of the year, soil, microbial inoculum and concentration, plant cultivar, seed origin and inoculation method were kept identical in both greenhouse and field experiments. Our results showed that the use of *R. irregularis* mixture (MRcoat) as a seed coating treatment has great potential to promote chickpea production, with significant increases in grain yield of plants grown in the field. Previous studies had already indicated that the inoculation of AM fungi can be used to improve biomass and productivity of chickpea (Zaidi et al., 2003; Erman et al., 2011; Pellegrino and Bedini, 2014), but with lower grain yield and harvest index, when compared to those obtained in plants inoculated with multiple *R. irregularis* isolates in our field trial. Harvest index is frequently used as an indicator of yield efficiency and consequently as a selection criterion for crop breeding (Fan et al., 2017). In our study, plants inoculated with the mixture of AM fungi were very effective and capable of producing a higher amount of grains with less shoot biomass. Contrary to previous reports (Weber et al., 1993; Erman et al., 2011), AM fungal inoculation did not influence the weight of 100 seeds.

Taking into account the grain yields obtained in our study (see Table 4.1), the producer price of chickpea in Portugal (1.10 USD Kg^{-1}, according to FAOSTAT, 2005) and the cost of seed coating (132.34 USD ha^{-1}, including materials and labor) we estimated the profit obtained for the

different treatments. Since there was no cost of seed coating in the control treatment, the obtained profit was 385.00 USD ha^{-1}. For the Rcoat and MRcoat treatments the estimated profits were 229.74 and 788.91 USD ha^{-1}, respectively. This shows that, despite the inoculation costs, choosing the right inoculum for seed coating can result in a substantial gain for the farmer.

Despite the common presence of AM fungi in agricultural soils, seed inoculation with selected isolates can increase plant root colonization and crop productivity (Lekberg and Koide, 2005; Lehmann et al., 2012; Pellegrino and Bedini, 2014). All field-grown plants, including non-inoculated controls, presented mycorrhizal root colonization, due to the ability of native fungi to colonize plant hosts. However, when compared to non-inoculated controls or plants inoculated with single fungal isolate, inoculation with multiple AM fungi increased root colonization and plant productivity. It is well known that the interactions among different AM fungal isolates can be synergistic, neutral or antagonistic (Koide, 2000; Jansa et al., 2008, 2009). Thus, inoculation of plants with non-native AM fungal isolates does not necessarily produce beneficial effects, as competition with native AM fungal species or even between selected species can occur. Soil physicochemical properties can also have a strong impact on the symbiotic relationship between plants and fungi (Kim et al., 2017). *R. irregularis* BEG140 was selected for this study due to the promising effects in increasing chickpea biomass and grain yield obtained in previous greenhouse trials (Oliveira et al., 2017b). Yet, the soil used in the above-mentioned experiment was sterilized (free of native AM fungi and remaining soil biota) and had different physicochemical characteristics from the soil used in this study, which had relatively high available P content. Our findings suggest that competition with native AM fungi or soil physicochemical status might have influenced the symbiotic relationship between *R. irregularis* BEG140 and chickpea, contrary to the MRcoat treatment where the combined used of *R. irregularis* isolates produced larger beneficial effects on plant growth. In general, it is considered that the combined use of soil microbes with different attributes provide extra benefit due to the combination and complementarity of different mechanisms of action (Malusà et al., 2016). Among other factors, the observed positive effects could be also due to the expansion of environmental niche for mycorrhiza functioning (Koide, 2000; Hart and Klironomos, 2003). Further investigation would be needed to evaluate which AM fungi isolates in the treatment MRcoat were active and responsible for the benefits.

Besides increasing plant productivity, inoculation of AM fungi can improve plant and/or seed nutrient content (Farzaneh et al., 2011; Pellegrino and Bedini, 2014; Oliveira et al., 2017b). However, our results showed no enhanced nutrient content of grains produced by inoculated plants. The exception was the higher grain crude fiber content in non-inoculated chickpea grown under field conditions, a fact that has been previously reported (Adewole and Ilesanmi, 2011; Masoero et al., 2018).

Despite the frequent demonstration of efficacy in laboratory and greenhouse experiments, the inconsistency of effectiveness or the lack of field data regarding AM fungi inoculation is still

one of the main restraints for its wide application (O'Callaghan, 2016; Thirkell et al., 2017; Lekberg and Helgason, 2018). According to the meta-analysis of Zhang et al. (2019) there is a bias favoring controlled conditions for AM fungi inoculation; laboratory studies including inoculated crops tend to lead to higher grain yield increase in comparison with those studies carried out in the field. Surprisingly, our work contrasts these data as it shows that the positive effect of multiple AM fungi inoculation is maximized under field conditions

Although direct comparison between results obtained under greenhouse and field conditions would be troublesome due to limiting aspects and interacting factors of the experimental procedure (e.g. constraining of roots within pots, root density/root system architecture and water requirements) (Poorter et al., 2012), both greenhouse and field scale are necessary and can be used as an indicator of potential positive effects. For instance, in field trials, Colla et al. (2015a) and Rouphael et al. (2017) successfully based their seed coating formulations on results obtained under greenhouse conditions where the AM fungal isolates had a positive influence on the growth, yield and nutrition of different plants species (zucchini, lettuce and winter wheat). Our results showed that the same treatment (MRcoat) was able to benefit chickpea performance both under greenhouse (plant SDW and seed weight increase) but especially under field conditions (pod and grain yield improvement). The above mentioned limiting aspects such as pot and root size/ depth or environmental factors (e.g. water, temperature) under greenhouse conditions, might have exerted a different influence on the AM fungi colonizing chickpea roots than in the field. This could have led to the observed differences in %RLC between treatments obtained under greenhouse and field conditions (Figure 4.2).

Conclusions

The selection of AM fungal isolates that relates to host plant and crop growing conditions is essential to achieve good mycorrhizal efficiency and to obtain economical profits from coated crops. Summarizing the main results obtained in this study, the application of multiple AM fungal isolates seemed to be a potential strategy to boost chickpea productivity, when compared to the inoculation of single AM isolate. Seed coating can be an appropriate tool to deliver AM fungi and the combined use of multiple isolates exhibited benefits for chickpea plants at different experimental scales, but the effect was especially relevant under field conditions. To our knowledge, this is the first field evidence of improved yield of chickpea inoculated with AM fungi via seed coating. Although greenhouse trials represent a prospective indication of microbial field-application potential, results are not necessarily representative in each case. In this sense, information provided by the combination of greenhouse and field trials is highly valuable and the simultaneous approach should be considered for further experimental designs. Integrating AM fungi into agricultural systems via seed coating in order to increase grain yield of crops is a potential valid approach for sustainable agriculture.

Chapter 5 – Using microbial seed coating for improved cowpea productivity under low-input agricultural system

This chapter was submitted as an original article to *Journal of Food Science and Agriculture*: Rocha I., Souza-Alonso P., Pereira G., Ma Y., Vosátka M., Freitas H. and Oliveira R. S. Microbial seed coating for improved cowpea productivity under low-input agricultural system

Abstract

Plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal (AM) fungi have the ability to enhance growth, fitness and quality of various agricultural crops, including cowpea. Yet, field trials confirming benefits of microbes in large-scale application using economically viable and efficient inoculation methods are still scarce. Microbial seed coating has a great potential for large-scale agriculture through the application of reduced amounts of PGPR and AM fungi inocula. Thus, in this study, the impact of seed coating with PGPR, *P. libanensis* TR1 and AM fungus, *R. irregularis* (single or multiple isolates) in grain yield and nutrient content of cowpea under low-input field conditions was evaluated. Seed coating with *P. libanensis* + multiple isolates of *R. irregularis* (coatPMR) resulted in significant increases in SDW (76%), pod and seeds number per plant (52 and 56%, respectively) and grain yield (56%), when compared with non-inoculated control plants. However, seed coating with *P. libanensis* + *R. irregularis* single-isolate (coatPR) did not influence cowpea grain yield. Grain lipid content was significantly higher (25%) in coatPMR plants in comparison with control. Higher soil organic matter and lower pH were observed in the coatPMR treatment. Our findings indicate that cowpea field productivity can be improved by seed coating with PGPR and AM fungi under low-input agricultural systems.

Keywords: Arbuscular mycorrhizal fungi; Agricultural sustainability; Cowpea; Field experiment; Plant growth promoting rhizobacteria; Seed coating

Introduction

Agricultural production has been, for the last decades, largely centered on unsustainable input of agrochemicals and water (FAO, 2018b). Currently, there is a demand for sustainable agricultural practices that safeguard food, air, water and soil quality, ensuring a safer environment for contemporary and future generations (Patil et al., 2014). Consequently, low-input and organic agriculture are gaining position worldwide as a way to preserve agro-ecosystem functionality and to reduce economic, environmental and health costs (Crowder et al., 2010; Postma-Blaauw et al., 2010, Pellegrino and Bedini, 2014; Reganold and Watcher, 2016). The evolution to more sustainable agriculture includes reducing or eliminating the use of non-renewable off-farm anthropoid inputs and giving high importance to the soil and its inhabitants in order to preserve and maintain ecosystem health (Gliessman, 2005; Royal Society, 2009).

Presently, there is a great interest in plant beneficial microbes such as arbuscular mycorrhizal (AM) fungi and plant growth promoting rhizobacteria (PGPR), as they play an important role in crop yield improvement and sustainable amelioration in agriculture (Nadeem et

al., 2014). Application of these microbes has been considered a key strategy to enhance legume production and quality while reducing the excessive use of chemical fertilizers and pesticides (Khan et al., 2010; Sindhu et al., 2010; Malusà et al., 2016; Singh et al, 2016). Cowpea is one of the most important edible grain legumes worldwide with great nutritional and nutraceutical properties and offers several agronomic, environmental and economic advantages for both developed and developing countries (Timko and Singh, 2008; Gonçalves et al., 2016; da Silva et al., 2018). Enhancement of biomass, grain yield and nutrient content in cowpea inoculated with AM fungi and PGPR has been previously demonstrated (Andrade et al., 2013; Oliveira et al., 2017). Yet, the lack of efficient inoculation methods of these microbes are a constraint for their large-scale application. Broadcasting of inocula of AM fungi and PGPR in open agricultural fields can be costly (Vosátka et al., 2012; O'Callaghan, 2016). Seed coating is considered a viable tool for precise and broad delivery of AM fungi and PGPR to different agricultural crops, and it has been explored in cowpea under greenhouse conditions (Ma et al., 2019; Rocha et al., in press). This inoculation method allows the application of low amounts of inocula in combination with other exogenous ingredients onto the seed surface, resulting in close plant-microbe contact at the early plant development stage. Despite the studies showing the great potential of AM fungi to enhance the nutritional status and productivity of various crops (Colla et al., 2015a; Cely et al., 2016; Oliveira et al., 2016b; Rouphael et al. 2017; Rocha et al., 2019), the application of AM fungi (single or in consortia) via seed coating is still scarce. Moreover, although greenhouse experiments provide important and useful data regarding the benefits of microbial inoculation, validation of microbial effects under field conditions across a range of environments is required (Ryan and Graham, 2018). In fact, the lack of consistency in field performance can be a major restraint for wider use of microbial seed coating (Nadeem et al., 2014; Thirkell et al., 2017). The aim of the present study was to evaluate the effects of seed coating with the PGPR *P. libanensis* and the AM fungus *R. irregularis* (single or multiple isolates) in cowpea productivity under low-input field conditions.

Materials and Methods

Seeds, Microbial Inocula and Coating

The seeds of cowpea [*Vigna unguiculata* (L.) Walp. cv. Fradel] used in this study were acquired from the INIAV collection. *P. libanensis* TR1 (GenBank accession no. KR051238), previously isolated from *Trifolium repens* rhizosphere existing in serpentine soils in Bragança (Portugal) was attained from the collection of the Centre for Functional Ecology, University of Coimbra (Ma et al., 2016). *P. libanensis* TR1 exhibited tolerance to heat (38 °C), salinity (8%) and severe drought (-1.5 Mpa) and it was tested positively for ACC-deaminase, indoleacetic acid (IAA), siderophores and ammonia production, phosphate solubilization and N fixation (Ma et al., 2019). In order to prepare inoculum for seed coating, the bacterial strain was grown in LB media

overnight at 28–30 °C and 200 rpm, according to Ma et al. (2016). Two AM fungal inocula were used: 1) a single fungal isolate of *R. irregularis (*BEG140) and 2) a mixture of *R. irregularis* isolates *(*BEG141, BEG236, DAOM 197198, KW and AS). Previously, all AM fungi inocula (provided by Symbiom Ltd, Czech Republic) were grown for 8 months with *Zea mays* L. as host plant, in a multispore pot culture with 1:1 (v/v) of zeolite and expanded clay. The AM Fungi inocula were cultivated as single isolate cultures and then equal part of inocula were mixed to prepare the *R. irregularis* mixture.

For the seed coating inoculation, cowpea seeds were previously immersed (for 45 min) in a *P. libanensis* solution with a concentration of 10^7 CFU ml^{-1}, then air-dried and dressed using a rotating pan (Scott et al., 1991). Cowpea seeds were firstly dusted with sieved (500 µm) *R. irregularis* inoculum and secondly with biochar (0.25% per seed weight) (Ecochar, Ibero Massa Florestal, Portugal), using a sticker solution of 2% gum arabic. Twenty AM fungal propagules were applied per seed (for both inocula), estimated according to the MPN method by Porter (1979). After coating, the final bacterial concentration was 10^6 CFU per coated seed. The CFU was estimated as described in Rocha et al., (2019).

Field Experimental Conditions and Design

The experiment was conducted in an agricultural field located in Elvas, Portugal (latitude 38°53'16.3 North, longitude 7°08'16.8 West), between June and September 2018. The soil had a clay texture and presented the following properties: 0.40 mS cm^{-1} electrical conductivity, 8.1 pH (1:2.5 w/v water), 0.08% N (Kjeldahl), >200 mg kg^{-1} extractable (Egner-Riehm) P, 138 mg kg^{-1} extractable (Egner-Riehm) K, 2768 mg kg^{-1} extractable (ammonium acetate) Ca and 417 mg kg^{-1} extractable (ammonium acetate) Mg. The field is normally used for cowpea and cereals (i.e. wheat and triticale) production in a crop rotation system. Before the beginning of the field experiment the site had been uncultivated for one year. The experimental design was based on three dissimilar inoculations: i) non-coated/inoculated seeds, control; ii) coated seeds with *P. libanensis* + *R. irregularis* BEG140, coatPR; and iii) coated seeds with *P. libanensis* + the above described mixture of *R. irregularis*, coatPMR. The experimental plot consisted of two rows of 3 m (with 10 seeds each and distanced by 60 cm) in a total of three repetitions per set of treatment organized in a split-plot randomized block. Seeds from different inoculation treatments were sown manually at 2-3 cm depth. No synthetic components were added to the soil (i.e. fertilizers or pesticides) before or during the experiment. Watering was done according to the plants requirement (enough to avoid water stress) using a drip irrigation system. During the experiment the temperature, RH and precipitation ranged from 13 to 26 °C, 42 to 78% and 0 to 8 mm, respectively.

Plant, Soil, and Grain Analyses

Approximately 83 DAS cowpea was harvested. Data regarding DAS to flowering (flowering 50% of the plants) and maturity (maturity of 50% of plants) were collected. Pods and seeds number per plant were counted. Seeds were weighted and plant shoots dried at 70 °C for 48 h and weighed. The grain yield (Kg ha^{-1}), weight of 100 seeds (average of 300 seeds from each treatment) and harvest index (the ratio of seed weight to SDW) were calculated.

At plant harvest, samples of the rhizosphere soil from the different treatments were collected and analyzed for pH (1:5 v/v), organic matter (dry combustion at 590°C), N (Kjeldahl method), P (Egner-Riehm) and K (Egner-Riehm) content. Samples of cowpea grains were dried (70 °C for 48 h), finely ground and used for the determination of crude protein, fiber, fat and ash content. Crude protein was analyzed by the Kjeldahl method according to ISO 20483:2006 and the protein was calculated as $N_{content}$ × 6.25. Crude fiber content was obtained by the ratio between the reduction in mass resulting from ashing of the dried digestion grain residue (acid and alkaline digestion) and the mass of the test sample, according to the method of intermediate filtration from the NP ISO 6865:2009. The determination of grain fat content was carried out using the extraction apparatus Soxtec System HT1043 with ether ethylic (NP 876:2001). The ash yield was obtained by the ratio between the difference in mass of ashing dish and incinerated grain residue, divided by the mass of the test sample (ISO 2171:2007).

Mycorrhizal Root Colonization

Roots were separated from shoots, washed, cut into 1-cm pieces and stained in a trypan blue solution in accordance with a modified Phillips and Hayman (1970) protocol (Oliveira et al., 2005). The grid-line intersect method (Giovannetti and Mosse, 1980) was used to estimate the % of RLC through observation of stained roots under a stereomicroscope (Leica EZ4 HD, Germany).

Bacterial Detection in Cowpea Rhizosphere

During harvest, plants were gently uprooted and 2 g of soils firmly adhered to roots (considered as rhizosphere soil) were collected and kept in small plastic bags. Bags were labelled, maintained in cold conditions (4 °C) during transport to the laboratory, where they were frozen at -20 °C. About 0.25 g of soil were used for DNA extraction using the NucleoSpin® soil extraction kit (Macherey-Nagel). Extracted DNA was stored at -20 °C. DNA purity was verified through absorbance (A260/A280) using a UV/visible spectrophotometer (NanoVue Plus, Biochrom). The presence of bacterial DNA was verified by amplification with the general primer pair 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'), targeted at the 16S rRNA of eubacteria. Reaction mix contained 2.5 µM of each primer, 12.5 µL of DSF Taq Master Mix (BIORON) (2.5 µL of buffer, 200 µM of dNTPs, 0.5 U of DFS-Taq

polimerase), and 1µL of template DNA in a final volume of 25 µL. A rapid amplification cycle was carried out with the following conditions: an initial denaturation step of 2 min at 95 °C, followed by 25 cycles of 20 s at 94 °C, 30 s at 57 °C, and 1 min at 72 °C, with a final extension step of 10 min at 72 °C.

Once the presence of bacteria was positively confirmed, the presence of *P. libanensis* TR1 in cowpea rhizosphere was studied using a nested-PCR approach. Initially, the specific primer pair GyrBF (5'-AGCATCAAGGTGCTGAAAGG-3') GyrBR (5'-GGTCATGATGATGATGTTGTG-3'), targeted at the gyrB gene (Agaras and Valverde, 2018), was used to detect the presence of *Pseudomonas* using the following PCR conditions: an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 57 °C, and 1 min at 72 °C, with a final extension step of 5 min at 72 °C. Reaction mix contained 2.5 µM of each primer, 12.5 µL of DSF Taq Master Mix (BIORON) (2.5 µL of buffer, 200 µM of dNTPs, 0.5 U of DFS-Taq polimerase), and 1µL of template DNA. Amplification produced a PCR product of approximately 1460 bp that was used for the specific identification of our strain. On the basis of the nucleotide sequence of the GyrB gene of *P. libanensis* TR1, we designed a set of specific primers targeted to unique regions within this sequence (Souza-Alonso et al. *in preparation*). In this study, two primer pairs designed to amplify specific regions of our strain were tested, including PsTR1a*For* (5'-CGACGACATCAGCATTATCA-3') and PsTR1b*Rev* (5'-CAGTGAGGATCAGTTCTTCG-3'), as well as PsTR1a*For* (5'-CGACGACATCAGCATTATCA-3') and PsTR1c*Rev* (5'-CGGACAGTGAGGATCAGTTC-3'). PCR products (5 µl) of the first round were further used for the nested-PCR using specific primers. In this case, reaction mix contained 2.5 µM of each primer, 10 µL of DSF Taq Master Mix (BIORON) (2.5 µL of buffer, 200 µM of dNTPs, 0.5 U of DFS-Taq polimerase), and 5µL of amplified DNA (using GyrB primers) as template. In both cases, amplification conditions were as follows: an initial denaturation step of 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 45 s at 52 °C, and 30 s at 72 °C, with a final extension step of 5 min at 72 °C.

In all cases, 5µl of the obtained PCR products were analyzed using agarose (1%) gel electrophoresis stained with GreenSafe Premium (NZYTech). DNA extracted from pure culture of *P. libanensis* TR1 served as positive control. Amplified DNA was visualized by GelDoc™ XR+ system with the Image Lab software (2.0.1, BIO-RAD).

Statistical Analysis

Data was analyzed using one-way analysis of variance (ANOVA) for each dependent variable versus the independent variable (inoculation), when normality and homogeneity of variances were confirmed. Duncan's multiple range test was use to compare treatment means, when F-values were significant ($P < 0.05$). When normality assumptions of parametric tests were not met, differences between groups of data were tested for significance using a non-parametric Kruskal–

Wallis test with a sequential Bonferroni correction for multiple comparisons, at $\alpha = 0.05/n$ with n the number of pairwise comparisons. All statistical analyses were carried out using the IBM SPSS Statistics 25.0 software package (IBM SPSS Statistics, USA).

Results

Seed coating did not hinder cowpea germination. Seeds took approximately 6 DAS to germinate, 50 DAS to flowering and 69 DAS to maturation, showing no significant differences between the different treatments.

Cowpea Productivity and Grain Quality

In general, seed coating with *P. libanensis* and the mixture of *R. irregularis* isolates (CoatPMR) increased cowpea productivity when compared with control treatment (Table 5.1). The coatPMR treatment showed a significant increase of 76% in SDW, 52% in the number of pods, 56% in the number of seeds per plant and grain yield. No differences were obtained in the weight of 100 seeds among treatments.

Table 5.1. Growth and productivity parameters of cowpea in different treatments [control, *Pseudomonas libanensis* + *Rhizophagus irregularis* (coatPR), *P. libanensis* + mixture of *R. irregularis* (coatPMR) under field conditions.

Treatment	SDW (g)	Number of pods per plant	Number of seeds per plant	Weight of individual seeds per plant (g)	Weight of 100 seeds (g)	Grain yield (kg ha^{-1})
Control	23.7±2.9 a	34.2±3.9 a	254.6±28.9 a	0.18±0.003 b	18.5±0.3	2537.2 a
CoatPR	31.2±3.3 ab	34.6±3.2 a	264.5±26.9 a	0.17±0.004 a	18.8±0.4	2489.3 a
CoatPMR	41.5±4.8 b	52.1±5.0 b	396.1±25.7 b	0.18±0.004 b	19.3±0.3	3952.2 b

Mean (± SE) followed by letters that indicate significant differences between treatments according to Duncan's multiple range test at $P < 0.05$. SDW, shoot dry weight.

CoatPR treatment had a negative influence in the seed individual weight when compared with control and CoatPMR, and lower harvest index percentage, comparing to CoatPMR (Table 5.1 and Figure 5.1).

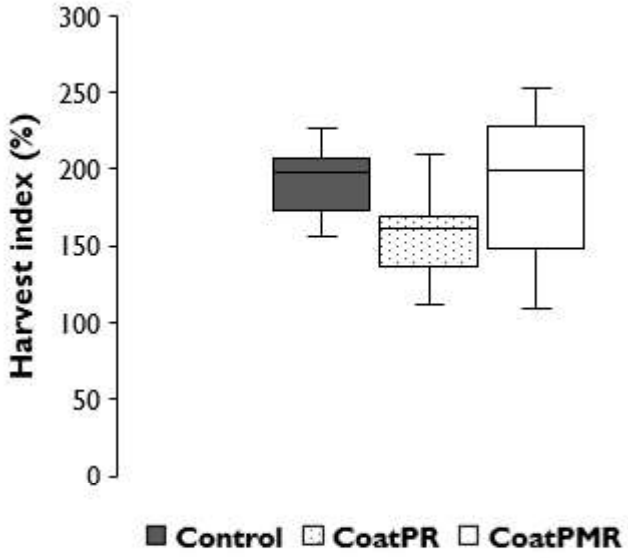

Figure 5.1. Harvest index (ratio of seeds to shoot dry weight) of cowpea in different inoculation treatments [control, *Pseudomonas libanensis* + *Rhizophagus irregularis* (coatPR), *P. libanensis* + mixture of *R. irregularis* (coatPMR).

A summary of cowpea harvesting productivity parameters among the different treatments is presented in Figure 5.2.

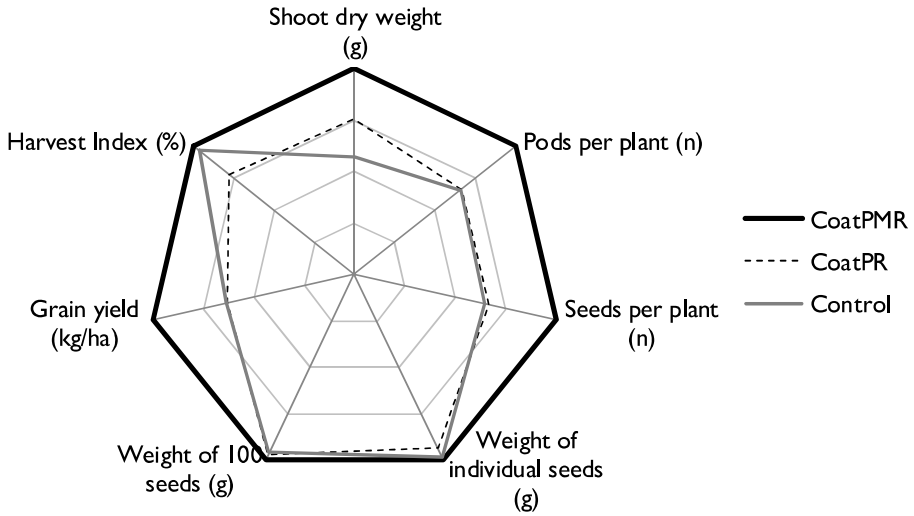

Figure 5.2. Representation of harvesting productivity parameters of cowpea in different inoculation treatments [control, coated with *Pseudomonas libanensis* + *Rhizophagus irregularis* (coatPR) or coated with *P. libanensis* + a mixture of different *R. irregularis* isolates (coatPMR)]. Radial graphs represent results relative to the higher value (indicated as 100%) for each productivity parameter.

Protein and crude fiber grain contents were not influenced by microbial coating (Table 5.2). In addition, fat content was significantly higher in seeds of coatPMR treatment in comparison to control and coatPR treatments. Ash content increased 6% in plants from the coatPR treatment.

Table 5.2. Cowpea grain content in different inoculation treatments [control, *Pseudomonas libanensis* + *Rhizophagus irregularis* (coatPR), *P. libanensis* and a mixture of *R. irregularis* isolates (coatPMR)]

Treatment	Crude protein	Fat (%)	Crude fiber (%)	Ash (%)
Control	20.8±0.3	1.0±0.03 a	3.8±0.1	3.2±0.05 a
CoatPR	20.8±0.5	1.1±0.04 a	3.9±0.1	3.4±0.06 b
CoatPMR	21.1±0.4	1.3±0.02 b	3.7±0.1	3.2±0.05 a

Mean (± SE) followed by letters that indicate significant differences between treatments according to Duncan's multiple range test and Kruskal-Wallis test at $P < 0.05$.

Soil Physicochemical Properties

Soil organic matter (SOM) and pH (Table 5.3) were significantly influenced by coatPMR inoculation. In terms of SOM, the values were higher in CoatPMR when compared with control. pH was lower in CoatPMR than that in control and coatPR. Both P and K had similar concentrations among different treatments.

Table 5.3. Soil physicochemical properties after plant growth.

Treatment	pH (H2O)	SOM (%)	N (%)	P (mg P2O5 kg-1)	K (mg K2O kg-1)
Control	8.2±0.03a	0.8±0.06a	0.07±0.00	358±27	175±3
CoatPR	8.2±0.03a	0.9±0.03ab	0.08±0.03	354±11	166±1
CoatPMR	8.1±0.00b	1.1±0.00b	0.08±0.03	355±5	173±4

Mean (± SE) followed by letters that indicate significant differences between treatments according to Duncan's multiple range test and Kruskal-Wallis test at $P < 0.05$. CoatPR (*Pseudomonas libanensis* + *Rhizophagus irregularis*), coatPMR (*P. libanensis* + mixture of *R. irregularis* isolates), nitrogen (N), soil organic matter (SOM), phosphorus (P) and potassium (K).

Microbial Colonization

Mycorrhizal colonization was significantly lower in coatPMR plants, which had a percentage of RLC of 29%, a substantial reduction in comparison with the 41% of colonization observed in control roots (Figure 5.3). Although coatPR treatment also showed a reduced percentage of RLC (37%), in this case the difference was not significant. After 83 days of plant growth, the presence of *P. libanensis* TR1 could not be confirmed in the roots and rhizosphere of coated cowpea. The presence of general bacteria and the *Pseudomonas* genus were positively confirmed in cowpea

rhizosphere by PCR detection using general primers (27f-1492r and GyrBF-GyrBR). However, the use of specific primers did not evidence the presence of our strain by the end of the assay.

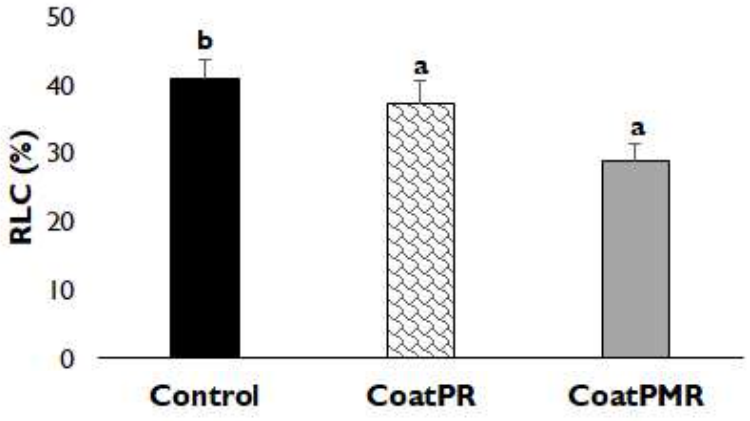

Figure 5.3. Mycorrhizal colonization, expressed as percentage of root length colonization (% RLC) in the roots of cowpea non-inoculated (control) and inoculated with *Pseudomonas libanensis* + *Rhizophagus irregularis* BEG140 (coatPR) or *Pseudomonas libanensis* + mixture of *R. irregularis* BEG141, BEG236, DAOM 197198, KW and AS (coatPMR) via seed coating. Columns are means ± 1 SE and letters indicate significant differences ($P < 0.05$) according to Duncan's Multiple Range test.

Discussion

Previous greenhouse studies reported benefits from AM fungi and PGPR either inoculated in the soil (Dastager et al., 2011; Omirou et al., 2016; Oliveira et al., 2017a, Oruru et al., 2018) or inoculated through seed coating (Rocha et al., in press) in cowpea performance. Nevertheless, there is still lack of information on microbial field performance particularly AM fungi, which can represent a significant barrier to up-scale microbial applications (Thirkell et al., 2017; Lekberg and Helgason, 2018). Our results indicate that seed coating with multiple AM fungal isolates and PGPR can be a promising tool to enhance cowpea productivity under field conditions, depending on the combination of beneficial microbes. According to Malusà et al. (2016), consortia of beneficial microbes can be more efficient than single-strain inoculants, due to the combination of various mechanisms of action of the different microorganisms present. In the field, plant global response should be considered multi-factorial and so, versatile consortia of microbes that harbor different characteristics can complement each other and become more functional. Yet, host specificity of microbial strains and growing conditions (e.g. soil properties, native microbiota, nutrition) can greatly affect the efficacy of beneficial microbes in improving the productivity of agricultural crops (Pellegrino and Bedini, 2014). In our study, coating with *P. libanensis* + multiple isolates of *R. irregularis* showed maximum cowpea growth and yield among treatments, while *P. libanensis* + *R. irregularis* single-isolate coating did not bring any advantage to cowpea performance. Despite having the same shoot biomass, plants from coatPR treatment produced fewer seeds than control, resulting in lower harvest index. The absence of beneficial effects of co-inoculation of *P. libanensis*

+ *R. irregularis* BEG140 coated onto cowpea seeds have already been shown in a greenhouse trial conducted by Ma et al. (2019). In the study, improvement in plant growth was only observed when *P. libanensis* was singly inoculated. Inoculation of a consortium of *P. libanensis* and *R. irregularis* showed no growth improvement on cowpea.

An estimation of the production cost of seed coating with microbial inoculants and the grain yield obtained in our study (see Table 5.1), showed that, choosing the right inoculum is crucial to obtain substantial profits for the farmer. Considering the producer price of chickpea in United States of America as 0.824 USD Kg^{-1} (FAOSTAT, 2005) and the cost of seed coating 154.67 USD ha^{-1} (estimation from the price of inocula and seed coating procedure, including coating materials and labor) the obtained profit for the farmer would be of 2090.65, 1896.13 and 3101,56 USD ha^{-1} for the control treatment, PRcoat and PMRcoat, respectively. This corresponds to an increase of 48% in profit brought by the use of seeds coated with the mixture of *R. irregularis* isolates.

Besides yield and growth promotion, PGPR and AM fungi can improve the nutritional quality of leaves, fruits or seeds of different crops (Berta et al., 2014; Hernández et al., 2016; Bona et al., 2017) and soil properties (Wu et al., 2008; Nadeem et al., 2014). In our study, lipid content and ash were increased by coatPMR and coatPR, respectively. Soil physicochemical properties were slightly modified after the growth of plants inoculated with *P. libanensis* + multiple AM fungi isolates. After plant harvest, soil pH was slightly reduced (0.1 units) and SOM experienced a significant increase (37.5%) when compared with control. It is well known that soil parameters such as SOM and pH strongly affect soil functions and nutrient bioavailability (Lehmann et al., 2014; Lehmann and Rillig, 2015). SOM contribute significantly to improving soil structure and aggregation, soil fertility, water retention and soil biodiversity. Usually, increases in SOM correspond to higher plant productivity (Lehmann and Kleber, 2015). The influence of inoculation of *P. libanensis* + multiple AM fungi isolates in soil physicochemical properties may have helped to increase cowpea productivity.

Not always high AM fungi root colonization corresponds to great crop yield (Marulanda et al., 2009; Ryan and Graham, 2018). Inoculation with AM fungi can significantly influence (e.g. promoting or hampering) the development of rhizobacteria and other fungi in plant roots and rhizosphere soils (Koch et al., 2011; Pellegrino and Bedini., 2014). Our results showed that cowpea coated with *P. libanensis* + multiple isolates of AM fungi had the highest productivity, but the lowest mycorrhizal root colonization. Possibly interspecific competition decreased the presence of native AM fungi, reducing overall colonization in cowpea roots. AM colonization can be highly dependent on their host, and in the symbiotic relationship plants can mediate competition to favor the high-quality interactions (Knegt et al., 2016). By providing different amounts of resources to fungal species, plants can affect the outcome of fungal competition (Pearson et al., 1993, Werner and Kiers, 2015). For instance, Bever et al. (2009) showed that plants can allocate resources in their roots in a selective way, giving preference and supporting the fungal species that provide more nutrients. Thus, either AM fungi mixture was more competitive than native AM fungi or a specific

AM fungal isolate presented better host specificity with cowpea, thereby prevailing over native. Consequently, it is possible that the reduction in the extent of mycorrhizal root colonization resulted in lower carbon cost for host plant and thus improved plant yield.

The presence of *P. libanensis* TR1 in the roots and rhizosphere of cowpea was not confirmed at the end of the field experiment. The decline of PGPR colonization in roots and rhizosphere of host plants can vary from high concentrations to low in a short time (Haas and Défago, 2005), depending on plant species (Landa et al., 2003). Nevertheless, the absence of the inoculated bacterial strain does not necessarily imply the absence of its effects as demonstrated by Rosas et al. (2009), who found that the concentration of the inoculated *P. aurantiaca* can decrease over time in the rhizosphere yet, with benefits in plant growth during the entire cycle of the crop.

Conclusions

To our knowledge, this is the first report of the improved yield of cowpea inoculated with AM fungi and PGPR via seed coating under field condition. In this study seed coating of multiple AM fungal isolates + *P. libanensis* improved cowpea productivity and seed quality in the field, when compared to non-inoculated plants. Apart from the increase in grains ash content, inoculation of single *R. irregularis* + *P. libanensis* did not influence cowpea performance, indicating that the right microbial combination is essential to achieve improved crop productivity. Substantial profit for farmers can be obtained through seed coating of cowpea with effective consortia of beneficial microbes. Seed coating can be a valid approach to deliver beneficial microbes into low-input and sustainable agricultural systems, aiming at improving soil properties, grain yield and quality.

Chapter 6 - General Conclusions and Future Work

In this final chapter the main findings of the previous data chapters are summarized and discussed in a general context. Future perspectives and potential research lines of microbial seed coating for sustainable agriculture are also presented.

General Conclusions

Agriculture is a sector of great importance that is currently facing various pressures (e.g. environmental degradation, climate change, water and energy scarcity). Thus, a proper and sustainable management of agricultural systems is required (FAO, 2018a). PBM, such as PGPR and AM fungi, will play a key role in the future of agriculture (Lesueur et al., 2016; Mahanty et al., 2017). Yet, despite the well-known and proven benefits of these beneficial microbes, few studies have been focused on feasible delivery systems to inoculate them in large-scale (Bhardwaj et al., 2014; O'Callaghan, 2016). In this thesis seed coating was proposed as a viable tool for PGPR and AM fungi inoculation under greenhouse and field conditions.

The literature review (Chapter 1) shows that the demand for microbial inoculants is rising and seed coating has the potential to be a cost-competitive and time-saving approach to deliver PBM. Yet, research on microbial seed coating still has some gaps that limit its broader use. Inoculant formulation is crucial for microbial survival onto the coated seeds yet, data on this topic is scarce. Studies comparing inoculant formulations (e.g. coating ingredients, cost) and seed coating with other inoculation methods are lacking. Inconsistency of results (e.g. biomass or yield increase, root mycorrhizal colonization, establishment of introduced microbes in the rhizosphere or in rhizoplane, nutrients enhancement) under field conditions is also a limitation. Finally, for better improvement of the technique, more clarity regarding the equipment and methodological details of microbial seed coating is required.

Seed Coating as a Microbial Deliver System

This thesis intended to explore the potential of seed coating as a delivery system for AM fungi and PGPR, using three major agricultural crops. Maize, cowpea and chickpea are some of the most cultivated and consumed crops worldwide and were selected due to their economic, nutritional and environmental importance in agriculture (Zerbe, 2015; Iqbal et al., 2006; da Silva et al., 2018). Seeds from these crops have different sizes, shapes and germination patterns, which mean that the seed coating process must be adjusted to the seed type in order to avoid negative impacts in germination and plant development. Results showed no adverse effect of seed coating in the germination of maize and chickpea seeds (Chapters 2 and 4, respectively) yet, cowpea seeds coated with inocula of *R. irregularis* (singly inoculated or in consortia with *P. putida*) took longer time to germinate when compared with seeds singly coated with *P. putida* and non-coated seeds (Chapter 3). The AM fungal inocula mixed with coating material hampered the germination which delayed cowpea development and adaptation to the growing conditions. Thereby, in Chapter 5, an alternative coating procedure was developed in order to overcome the undesirable delay in cowpea germination. Hence, improving seed coating and creating new formulations according to the target crop was shown to be a crucial task to attain a feasible inoculation method.

As expected seed coating reduced the amount of AM fungi inoculum needed for an effective inoculation (Chapter 2). The comparison between seed coating and direct soil inoculation of *R. irregularis* BEG140 showed similar root colonization, proving that the use of minor amounts of inoculum through seed coating is viable. Similar results were published by researchers involved in this PhD program (Oliveira et al., 2016b) using wheat seeds also coated with *R. irregularis* BEG140 and the same coating procedure. Wheat seeds are smaller and thinner than maize seeds and yet, the technique was successful, showing the potential of seed coating for AM fungi inoculation. Despite the delay in germination, AM fungi were successfully delivered by seed coating to cowpea seeds (Chapter 3). In this study, sterile soil was used, and only plants inoculated with AM fungi presented root AM colonization. On the other hand, in Chapters 4 and 5, notwithstanding the use of non-sterile agricultural soil (with the presence of native AM fungi), significant differences after inoculation were detected. Under field conditions, root colonization of chickpea coated with a mixture of *R. irregularis* isolates was higher than that of plants inoculated with a single-isolate of *R. irregularis* and non-inoculated controls (Chapter 4). The greater root colonization corresponded to enhanced crop productivity (increased number of pods, seeds and grain yield) for chickpea. The same mixture of AM fungal isolates was coated onto cowpea seeds together with *P. libanensis* which also resulted in improved plant productivity (Chapter 5). However, mycorrhizal colonization of cowpea coated with *P. libanensis* + multiple isolates of *R. irregularis* was significantly lower when compared to non-inoculated seeds, and seeds coated with *P. libanensis* + a single isolate of *R. irregularis*. The differences between AM root colonization in these two studies could be related to the host plant, the specificity of the AM fungal isolate, competition with native AM fungal species and soil physicochemical properties (Pellegrino et al., 2014; Kim et al., 2017). These results indicate that the selection of PBM must be adjusted to the growing conditions and target plant.

The presence of the PGPR *P. fluorescens* (Chapter 2) and *P. libanensis* (Chapter 5) in the rhizosphere and roots of maize and cowpea, respectively, could not be confirmed by Real Time-PCR and PCR detection, respectively, at plant harvest. Yet, despite the absence of the bacteria, prior to sowing, coated seeds had a concentration of 10^5 and 10^6 CFU per seed for *P. fluorescens* and *P. putida,* respectively, amounts considered sufficient for successful colonization (Weller, 1983; Tang et al., 1995; Landa et al., 2003). The decline of inoculated PGPR in the rhizosphere could have been related with several factors, including the ability of the bacteria to compete for nutrients with native microbial community, to adapt to soil conditions (e.g. physicochemical properties, water availability), and the compatibility with the plant host species, since plants can promote the presence of some bacteria in detriment of others (Landa et al., 2003; Farrar et al., 2014). Overall, these findings highlight the need to monitor the survival of inoculated PGPR over time and not only at plant harvest.

Microbial Influence According to Fertilization and Water Deficit Regime

About 60% or more of the total applied synthetic fertilizers is lost and not used by plants (Bhardwaj et al., 2014). PGPR and AM fungi can assist plants by increasing nutrients availability and/or by preventing nutrients from leaching out (Adesemoye and Kloepper, 2009). One of the goals of this thesis was to evaluate the role of AM fungi and PGPR inoculation via seed coating in reducing the input of chemical fertilizers. Maize coated with microbial inoculants displayed enhanced shoot concentration of macro and micronutrients, under different fertilization regimes (Chapter 2). N, P, K, Mg and Zn contents in maize shoots were significantly increased in plants obtained from seeds coated with *R. irregularis*, particularly in treatments where fertilization was reduced or absent. On the other hand, plants coated with *P. fluorescens* presented most of the nutritional increments when full fertilization was applied. No biomass enhancement was observed in plants inoculated with *P. fluorescens* or *R. irregularis* (single or mixed isolates). These results confirm the utility of PBM to increase nutrient uptake of crops and improve their nutritional value. PBM seed coating can be of great interest in nutrient management strategies, especially for low agrochemical input systems and sustainable agriculture.

Besides nutrients, water is essential for plant development and growth. Water scarcity is a major constraint for crop productivity, and tends to be intensified by the future climate change scenario (Misra et al., 2014). The role of microbial inoculants in increasing plant tolerance to drought is gaining relevance (Li et al., 2014; Vurukunda et al., 2016; Oliveira et al., 2017a; b). The results presented in Chapter 3 showed a general positive effect of AM fungi on cowpea regarding nutrient uptake, leaf pigments content and leaf gas exchange parameters mostly obtained without water deficit. However, there was no increase in plant productivity of inoculated plants. This could be associated with the sink of carbohydrates of the fungal mycelium and the delay on seedling emergence of AM plants. Moreover, the fact that AM fungal treatments took longer to germinate could have negatively influenced their relationship with the host plant by affecting the C and nutrient balance, which can be essential for crop yield and adaptation to water stress. On the other hand, when singly coated, *P. putida* increased cowpea root biomass and seed yield under moderate water deficit. The results obtained in Chapter 3 highlight the importance of selecting microbial inoculants according to their ability to confer drought tolerance to the target-plant.

Microbial Seed Coating for Field Application

Data regarding benefits of PGPR and AM fungi to crops in real agricultural field conditions is essential to evaluate the viability of large-scale applications. Especially, for seeds coated with AM fungi, data on their benefits for crop production, under field conditions, are very scarce. In Chapters 4 and 5 field trials showed that selection of AM fungal isolates can be determinant for improving crop productivity. Application of multiple AM fungal isolates was considered the best strategy to boost chickpea and cowpea productivity, when compared to single AM isolate inoculation and non-inoculated controls. The use of multiple isolates exhibited benefits under both

greenhouse and field conditions (Chapter 4). Inoculation may influence soil physicochemical properties which can consequently improve cowpea productivity (Chapter 5). The findings indicate that PBM can have an indirect contribution to plants, by altering soil conditions in their behalf.

An estimation of the production cost of seed coating with microbial inoculants, showed that, seed coating can be a feasible method for field-delivering PBM with significant economic gains (Chapters 4 and 5). Nevertheless, the profits are only possible if the applied inocula are best adapted to the farmer's needs (target crops, growing conditions and agricultural practices). These studies increased the knowledge on AM fungi performance under field conditions and were the first evidence of improved yield of chickpea and cowpea under field conditions through seed coating inoculation of AM fungi.

Briefly, this PhD thesis contributed to a better understanding of the role of microbial seed coating in agriculture, by reviewing the work developed so far and by studying seed coating inoculation on several agricultural crops, with various microbial combinations, under greenhouse and field conditions. Results confirmed that seed coating is capable to efficiently deliver low amounts of AM fungi inocula, when compared with direct soil inoculation and assure sufficient root colonization. Therefore, seeds coating has potential to reduce inoculation costs and application efforts for farmers (Chapter 2). Coating formulations can be critical for seed germination, and thus it is important to improve the seed coating technique, not only to be best adapted to seed germination, but also to ensure the survival of the inoculated PBM (Chapter 3). Overall, the obtained data confirms the potential of microbial inoculants in improving plant nutritional status, resistance to water deficit and crop productivity under greenhouse and field conditions. Yet, results also show that benefits can be highly dependent on the selection and combination of microbial inoculants and crop growing conditions (Chapters 2, 3, 4 and 5). Furthermore, field studies are paramount to corroborate the benefits of microbial seed coating and validate its feasibility for large-scale applications. With the present demand for sustainable practices, inoculation of AM fungi and PGPR via seed coating can be regarded as a path to sustainable agriculture.

Future Work

As a tool with great potential to deliver microbial inoculants, such as PGPR and AM fungi, seed coating can be further explored in order to allow a wider application and integration in agricultural management strategies. Therefore, the following research lines are proposed for the progress of microbial seed coating:

1. *Development of new microbial seed coating formulations*. Use of microbial inoculants based on native isolates that are best adapted to the local edaphoclimatic conditions (e.g.

temperature, precipitation, soil properties) and agricultural practices (e.g. irrigation, fertilization). Improvement of viability of inocula for seed coating and reducing the inoculant-cost using in vitro propagules of AM fungi. Testing novel formulations with low-cost and alternative coating materials (e.g. binders, fillers/carriers) such as compost and residues from forestry and agriculture for more environmentally friendly and economical ingredients.

2. *Evaluation of microbial seed coating under various agricultural scenarios*. Comparison between seed coating and other inoculation methods in conventional and organic agricultural fields by testing the influences of practices such as inter-cropping, reduced fertilization or irrigation. Further testing of microbial seed coating under stress conditions, such as drought and salinity, as well as the related mechanisms used by PBM to increase stress tolerance and crop yield.
3. *Improvement of microbial detection*. Greenhouse and field studies for evaluating the survival of PGPR after inoculation, namely the presence in the rhizosphere/roots of the target-plant over time. Besides detection, it would be of great interest to understand the impact of the presence/concentration of the bacteria in the plant, by collecting samples of rhizosphere/roots while performing simultaneous measurements of growth (e.g. shoot dry weight, number of seeds per plant) and physiological (e.g. photosynthesis, respiration, plant hormone functions) parameters, in different stages of the plant cycle. Further, the impact of AM fungi and PGPR inoculation on the native microbial community should be evaluated.
4. *Evaluation of economic feasibility of seed coating*. Studies comprising costs and gains for farmers (e.g. increased yield, reduction of fertilizers/pesticides and irrigation costs) are required for further assessing profits of seed coating.

PGPR and AM fungi have certainly a place in the future of sustainable agriculture. Therefore, it is important to assure that they are successfully applied. Seed coating is a tool that, with the right development and investment, may allow the application of these beneficial microbes at large-scale in sustainable agricultural systems.

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