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BIOLOGICAL CONTROL OF RICE BLAST DISEASE CAUSED BY *Pyricularia oryzae* WITH *Bacillus amyloliquefaciens*, *Epicoccum nigrum,* AND *Penicillium citrinum*

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Dedicated to

My

Beloved Family

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Having unflinching faith in to “omnipotent creator” for his never-ending blessing, it is a great pleasure for the author to express his profound gratitude to his respected parents, who digested much hardship inspiring for prosecuting his education, thereby receiving proper education. According to someone "A service loses its grace by publishing it too much". But how can one remain silent when an act done imposes respect and recognition?

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RESUMO

Os objetivos dos experimentos foram avaliar o potencial de controle biológico de *Bacillus amyloliquefaciens, Epicoccum nigrum* e *Penicillium citrinum* para o controle da brusone do arroz causada por *Pyricularia oryzae* na cultivar de arroz (*Oryza sativa* L.) BRSMG Caçula; na capacidade de estimular o crescimento e a produtividade das plantas, bem como analisar a capacidade para estimular os mecanismos de defesa da planta por meio de análises microscópicas e moleculares. Os experimentos foram realizados em casa de vegetação. As avaliações foram baseadas na incidência e severidade da brusone do arroz, bem como na altura das plantas, no número de perfilhos e de panículas e no peso dos grãos de arroz. O microscópio confocal a laser (CLM), o microscópio eletrônico de varredura (SEM) e o microscópio Epi-fluorescência (EFM) foram utilizados para analisar a interação das plantas de arroz com os endófitos microbianos. Solo e sementes de arroz foram inoculados com *Bacillus* BMH e as sementes foram pulverizadas com os isolados 10965 e IA25 de *P. oryzae*. *Bacillus* BMH reduziu a severidade da brusone do arroz em 53,1%, 50% e 37,5% quando aplicado no solo ou via tratamento foliar preventivo e mistura de patógeno-BMH, respectivamente. A altura das plantas de arroz foi maior no tratamento com *Bacillus* BMH aplicado em solo (28,5 cm) em comparação ao tratamento controle (25,7 cm) aos 63 DAS. Da mesma forma, o número de perfilho foi maior nas plantas desenvolvidas no solo tratado com *Bacillus* BMH (12,6) em comparação com o controle (9,13). O peso dos grãos foi de 10,2 g para tratamentos de solo com *Bacillus* BMH (escrever quais) contra 7,1 g para o não tratado. A colonização de tecidos vasculares das raízes e das folhas de arroz por células de *Bacillus* BMH foi observada em CLM e SEM indicando o seu endofitismo. O tratamento de sementes de *Bacillus* BMH estimulou mecanismos de defesa das plantas contra a brusone por meio da regulação positiva dos genes β-1,3-glucanases (OsGLN1), OsPR1a e OsWRKY28 do arroz. A embebição de sementes de *E. nigrum* e a pulverização nas folhas reduziram preventivamente a incidência de brusone em 41,7% a 31,2%, respectivamente, e a severidade em 54,5% a 34,9%. O tratamento das sementes com *E. nigrum* aumentou o número de perfilho para 8,3 contra 5,9 no tratamento controle. As imagens em SEM e EFM mostraram a colonização da superfície das raízes do arroz por *E. nigrum*. *Penicillium citrinum* GP1 e GP3, tanto em tratamento de sementes do consórcio como pulverizados

nas folhas, diminuíram a severidade do brusone em 33,3% a 37,4%, respectivamente. A mistura dos isolados de *P. citrinum* aumentou o número de perfilhos do arroz com 11,25 contra 10,17 no controle, respectivamente. O uso individual ou combinado desses isolados não teve efeito na altura da planta, nem no número de panículas e no rendimento das plantas em comparação com as não tratadas. Ambos os isolados colonizaram os tecidos das raízes do arroz. Dois isolados de *Epicoccum* (de videira e milho), usados neste estudo, foram identificados como *E. nigrum* com base nas regiões ITS, RPB2, β-TUB2 e LSU de homologia de sequência de rDNA e análises filogenéticas. Da mesma forma, os dois *Penicillium* da videira (GP1 e GP3) foram identificados como *P. citrinum* com base nas regiões RPB2, β-TUB2 e LSU da homologia de sequências de rDNA e análises filogenéticas. Os experimentos em casa de vegetação mostraram que *Bacillus* BMH, *E. nigrum* e *P. citrinum* diminuíram a severidade da brusone, bem como estimularam o crescimento das plantas. Os efeitos benéficos sugerem que esses agentes de biocontrole apresentam potencial para serem utilizados como biofungicidas e biofertilizantes na cultura do arroz.

Palavras-chave: *Pyricularia oryzae, Bacillus amyloliquefaciens, Epicoccum nigrum, Penicillium citrinum*, biocontrole, brusone, promoção de crescimento, microscopia, indução de resistência.

ABSTRACT

The objectives of the experiments were to evaluate the biological control potential of *Bacillus amyloliquefaciens, Epicoccum nigrum,* and *Penicillium citrinum* for the control of rice blast caused by *Pyricularia oryzae* in the rice cultivar (*Oryza sativa* L.) BRSMG Caçula, in the ability to enhance plant growth and productivity, as well as to analyze the ability to stimulate plant defense mechanisms through microscopic and molecular analyses. The experiments were carried out in a greenhouse. Evaluations were based on the incidence and severity of rice blast, not only but also plant height, several tillers, panicles, and rice grain weight. Confocal laser microscope (CLM), scanning electron microscope (SEM), and Epi-fluorescence microscope (EFM) were used to analyze the interaction of rice plants with microbial biocontrol agents (BCAs). Soil and rice seeds were inoculated with *Bacillus* BMH and the seeds were sprayed with *P. oryzae* isolates 10965 and IA25. *Bacillus* BMH reduced rice blast severity by 53.1%, 50% and 37.5% when applied to soil or via preventive foliar treatment and pathogen-BMH mixture, respectively. The height of rice plants was higher in the treatment with Bacillus BMH applied in soil (28.5 cm) compared to the control treatment (25.7 cm) at 63 DAS. Likewise, the number of tillers was higher in plants grown in soil treated with *Bacillus* BMH (12.6) compared to the control (9.13). The grain weight was 10.2 g for soil treatments with *Bacillus* BMH (write which ones) versus 7.1 g for the untreated. Colonization of vascular tissues of rice roots and leaves by *Bacillus* BMH cells was observed in CLM and SEM indicating its endophytism. *Bacillus* BMH seed treatment stimulated plant defense mechanisms against blast through upregulation of rice β-1,3-glucanases (OsGLN1), OsPR1a, and OsWRKY28 genes. *E. nigrum* seed soaking and spraying on leaves preventively reduced blast incidence by 41.7% to 31.2%, respectively, and severity by 54.5% to 34.9%. Seed treatment with *E. nigrum* increased the number of tillers to 8.3 against 5.9 in the control treatment. SEM and EFM images showed the rice root surface colonization by *E. nigrum*. *Penicillium citrinum* GP1 and GP3 consortium in seed drenching treatment and sprayed on leaves, reduced blast severity by 33.3% to 37.4%, respectively. The consortium of *P. citrinum* isolates increased the number of rice tillers by 11.25 versus 10.17 in the control, respectively. The individual or combined use of these isolates did not affect plant height, panicle number, and plant yield compared to untreated plants. Both

isolates colonized rice root tissues. Two strains of *Epicoccum* (from grapevine and maize), used in this study were identified as *E. nigrum* based on ITS, RPB2, β-TUB2, and LSU regions of rDNA sequence homology and phylogenetic analyses. In the same way, the two *Penicillium* from grapevine (GP1 and GP3) were identified as *P. citrinum* based on RPB2, β-TUB2, and LSU regions of rDNA sequence homology and phylogenetic analyses. The greenhouse experiments showed that *Bacillus* BMH, *E. nigrum* and *P. citrinum* reduced blast severity as well as stimulated plant growth. The beneficial effects suggest that these biocontrol agents have the potential to be used as biofungicides and biofertilizers in rice cultivation.

Keywords: *Pyricularia oryzae*, *Bacillus amyloliquefaciens*, *Epicoccum nigrum*, *Penicillium citrinum*, biocontrol, blast, growth promotion, microscopy, resistance induction.

PREAMBLE

This thesis, which is part of obtaining the degree of Doctor of Agronomy/Phytopathology, was carried out at the Electron Microscopy and Ultrastructural Analyze Laboratory (LME) of the Department of Phytopathology (DFP) of the Federal University of Lavras (UFLA) Minas Gerais, Brazil. All of the research work for this thesis presented was exclusively carried out in this laboratory and greenhouse.

It is part of the South-South Technical Cooperation Program launched by the Brazilian Cooperation Agency (ABC). More precisely between the Federal University of Lavras (UFLA) and the Institute of Rural Economy (IRE) of Mali, through a scholarship offered by UFLA's Department of Phytopathology (DFP).

Prior the classroom courses were spread over a three-semester period. This dissertation, which reports on all of the work carried out during this thesis, is divided into three parts. The first concerns the introduction and bibliographical synthesis, placing the subject in its context, arising from its objectives and enabling the thesis subject to be placed in its scientific context respectively. The second described the experimental research, also divided into three chapters. The first chapter describes the control of blast disease with *B. amyloliquefaciens* isolate BMH with the abstract, introduction, the development of the methods necessary to carry out the study, the results, discussion, conclusion, acknowledgement, and references. The second chapter described the contains of the article on *E. nigrum* with the same subtitle as the *Bacillus* manuscript. The third contains the results of *P. citrinum* experiments. Finally, at the end of this dissertation, there is a general conclusion and the appendix's, which contain the supplementary research on the test of three rice genotypes growth promotion with *E. nigrum,* the details of the protocols used (except those already described in the publications).

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LIST OF ABBREVIATIONS

AUDPC: area under disease progress curve

BAS: biotrophy-associated secreted

BCA: Biological Control Agent

BIC: biotrophic interfacial complex

BMH: Bacteria Markos Humberto

CAT: Catalase

CEBiP: chitin elicitor binding protein

CLM: Confocal Laser Microscopy

CNPq; National Council for Scientific and Technological Development

DAI: Day after inoculation

DAS: Day after sowing

dNTP: deoxynucleotide triphosphate

EFM: Epi-Fluorescent Microscopy

ET: Ethylene

ETS: effector-triggered susceptibility

FINEP: Funding Authority for Studies and Projects

GP: Grapevine

MAMP: microbe-associated molecular pattern

MAPK: mitogen-activated protein kinase

NLR: nucleotide-binding site leucine-rich repeat

PAL: Phenylalanine Ammonia Lyase

PAMP: pathogen-associated molecular patterns

PGPM: Plant Growth Promotion microbe

PGPR: Plant Growth Promotion Rhizobacteria

Pmk: mitogen-activated protein kinase

POX: Peroxidases

PPO: Polyphenol Oxidases

PRR: pattern-recognition receptors

PTI: PAMP-triggered immunity

QS: Quorum Sensing

RLK: receptor-like kinases

rpm: revolutions per minute

SA: Salicylic Acid

SEM: Scanning Electron Microscopy

Slp1: secreting Secreted LysM Protein1

SOD: Superoxide dismutase

LIST OF ACRONYMS

ABC: Brazilian Cooperation Agency
CAPES: Foundation for the scholarship granted
DFP: Department of Phytopathology
LME: Laboratory of Electron Microscopy
UFLA: Federal University of Lavras

LIST OF SYMBOLS

cm: centimeter
ha: hectare
h: hour
IH: invasive hyphae
IER: Institute of Rural Economy
IPM: Integrated Pest Management
ISR: Systemic Induced Resistance
JA: Jasmonic Acid
kg: kilogram
km: kilometer
l: liter
m: meter
m^2: square meter
mg: milligram
min: minute
mm: millimeter
ml: milliliter
°C: degree Celsius
e.g.: for example (exempli gratia)
g/l: gram/ liter
%: Percentage
µg: microgram
µl: microliter
bp: Base pair

CONTENTS

PART I- GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Rice (*Oryza sativa* L.) is the second most important cereal cultivated and the third consumed and exported to the world after wheat and maize. It is the staple food of more than half of humanity mainly in Asia, South America and Africa (FAGERIA et al. 2014). Rice is cultivated on all the continents except Antarctica (FAGERIA et al. 2014). According to Shaheen et al. (2022), world rice production in 2021 was 755,474 million tons from 510,072 million ha. The contribution of Brazil to world rice production was about 12 million tons of paddy rice (FAGERIA et al. 2014). In Brazil, there are two main rice-growing ecosystems of which as upland rice and irrigated lowland rice with a cultivated area of about 2.62 million hectares (FAGERIA et al. 2014). Brazil is the eighth producer of rice in general ranking with 12.3 million tons in 2017, and the major upland rice producer worldwide (FAO 2017; NASCIMENTO et al. 2019; SENA et al., 2013). According to this statistic, Brazil is placed this year in tenth place in the world with 10,370 million tons from 8,516 million ha worldwide (SHAHEEN et al. 2022).

In Africa, rice occupies a preponderant place in the food diet and represents more than 25% of the total cereals consumed behind the maize (MENDEZ; BAUER, 2019). Mali, an agro-pastoral country with an economy strongly supported by the primary sector, represents 44% of the Gross domestic product (GDP) and occupies 83.4% of the assets, whose rice represents 25% of all other cereals (GUINDO, 2017, p. 77). It is grown in five different ecosystems of which it contributes about 5% of GDP, and its consumption represents more than 30% of total cereal (DIARRA et al. 2014).

Due to the events of the green revolution of the 1960s, based on the use of efficient genotypes demand water and fertiliser, and increased trade, there has been an increase in crop diseases (LEPOIVRE, 2003, p. 427). It had been reported by Buchanan et al. (2015 p. 1207) that, crop yield losses each year owing to the pests and pathogens estimated 25 to 35% worldwide necessitating 30 billion USD for agrochemical products by year. According to Thakur (2016), out of 31% to 42% of total losses due to all kinds of pests (pathogens, insects and weeds), 14.1% are caused by diseases and the total annual loss is due to disease is about 220 million US dollars. On rice, more than 70 different diseases caused by fungi, bacteria, viruses, and nematodes have been listed (ZHANG et al. 2009). Among them, there is *Pyricularia oryzae* Cavara (syn. *Magnaporthe*

oryzae B. Couch) caused blast disease (CASTROAGUDÍN et al., 2016). This filamentous, heterothallic ascomycete species collectively causes disease on many different host species in the *Poaceae* family, including rice (*Oryza sativa*) and wheat (*Triticum aestivum*) (CASTROAGUDÍN et al., 2016; GLADIEUX et al., 2018; HOWARD; VALENT, 1996). *Pyricularia* is a multi-host pathogen that infects more than 50 other species of grasses (GLADIEUX et al., 2018). Rice blast disease caused by *P. oryzae* is a cosmopolitan disease (AGARWAL et al. 1994; BETTS, 2007; OU, 1980) that has been found in more than 85 countries (He et al. 2019). It is particularly more detrimental to upland rice (HARINJAKA; MATHILDE, 2012; NGUYEN et al., 2016). The annual losses of rice caused by *P. oryzae* may vary from 10% to 30% of the production, or even 100% with the very sensitive cultivars (AGRIOS, 2005, p. 922; RAVELOSON et al, 2013; SPENCE et al. 2014). This seed-borne and foliar pathogen can spread around the world through the movement of seed (GLADIEUX et al. 2018). It may produce lesions on leaves (leaf blast), leaf collars (collar blast), culms, culm nodes, neck nodes (neck rot), panicles (panicle blast) even the root (ASIBI et al. 2019; RAAIJMAKERS et al., 2013; SESMA; OSBOURN 2004; ZHANG et al. 2016).

Given the great economic importance of rice and the devastating nature of blast disease, various management strategies have been adopted. Among these strategies, the use of pesticides, resistant cultivars, the controlled use of nitrogen fertilizers, and prophylactic methods have long been used to reduce blast disease (POOJA; KATOCH, 2014). In general, the application of fungicides and the use of resistant cultivars although effective (TEBEEST et al. 2015), are shown their limit because of the pathogen's ability to evolve to overcome resistance genes (NGUYEN et al. 2016; TEBEEST et al., 2015). This fungus has caused the breakdown of resistance conferred by newly developed commercial cultivars (CHUMA et al. 2011). The continuous use of chemical fungicide lead to environmental threat (risks of toxicity, ecotoxicity), and the appearance of resistant strains of the pathogen (RAVELOSON et al., 2013). At this crucial time, there is a need to seek crop production strategies more sustainable, advantageous, respectful of the environment, and allow crops to produce well (DUBEY et al. 2015). Therefore, the main challenge of agriculture is the production of high quantity and quality of food, safe, and affordable for a growing world population. Biological Control Agents (BCAs) are considered to be one of the best strategies, a better alternative to conventional agriculture and a viable solution to current food challenges. The

use of these BCAs, due to their long-term mechanisms for controlling pathogens and promoting plant growth, is increasingly widespread in the agricultural industry (SHAH et al. 2021). Their enormous biological diversity, associated with their ability to synthesize several secondary metabolites to control pathogens, promote plant growth (PGP), and enhance plant fitness has prompted research on these BCAs (SCHIFFERS, 2011, p. 126). Their nature allows their application both in organic and conventional farming (MORICCA et al. 2005). In this way, managing crops using BCAs is a promising and environmentally friendly approach that can be used alone or in combination with other approaches in the context of Integrated Pest Management (IPM) in sustainable agriculture (DUBEY et al. 2015).

In this study, we highlight the contribution of members of beneficial endophytic BCAs, as a strategy for the control of rice blast disease, improvement of plant growth, and productivity. As is known, viruses, bacteria, fungi, and oomycetes antagonistic are used to control a wide range of plant pathogens and are exploited as biopesticides (KÖHL et al. 2019). Thus, bacteria and fungi are viable BCAs, which have been used in different disease management on various crops (REY et al. 2008; SENA et al. 2013; MOYA et al., 2016).

Bacillus genus is a gram-positive bacterium that has a unique ability to grow rapidly, is resistant to adverse environmental conditions and has a broad spectrum of BCA ability (SHAFI et al. 2017). It has been found that soil-inhabiting exist as epiphytes and endophytes in the spermosphere and rhizosphere (BERIĆ et al. 2012). Many species of *Bacillus* also produce secondary metabolites and have been found to show antifungal activity against different phytopathogens (ARGUELLES-ARIAS et al. 2009). According to Saranraj (2014, p. 1265-1277), *Bacillus* is one of the most abundant genus in the rhizosphere, having PGPR activity, resulting in the synthesis of some metabolites like auxin, cytokinin and gibberellins. Rhizobacteria are bacteria from the rhizosphere that can colonize and develop plant root systems in the presence of competing soil microflora (KLOEPPER et al. 1999). Also, the volatile compounds produced by *B. subtilis* may play an important role in plant growth promotion, and the activation of plant defense mechanisms by triggering the induced systemic resistance (ISR) in plants (COMPANT et al. 2005; SHAFI et al. 2017).

Epicoccum spp. of the order Moniliales and family of Dematiaceae is a ubiquitous fungus, routinely found in air, soil, and on decaying vegetation (BRAGA et al. 2018). It is used as a biocontrol agent against numerous phytopathogenic fungi and for its ability to produce many secondary metabolites, such as antioxidant, and antimicrobial compounds (LIMA FÁVARO et al. 2011; PASCUAL, et al. 2000). According to Li et al. (2013), the production of the antifungal compound, the production of large spores with large amounts of stored nutrients, and the increased ability to survive unfavorable climatic conditions make *Epicoccum* an attractive candidate as a bioagent. Some studies suggested that *E. nigrum* is considered an epiphytic and endophytic fungus (FÁVARO et al. 2011) possessing melanized conidia which increases its tolerance to ultraviolet (UV) rays and can colonize the phylloplane (LARENA et al. 2019). It is considered a promising source of a wide variety of vital metabolites such as alkaloids, flavonoids, phenols, steroids and terpenoids (NISA et al. 2015).

The genus of Penicillium contains a large number of species is of well-recognized and widely distributed fungi present in the air, soil, vegetation, and indoor environments (Tsang et al. 2018). Species of this genus are decomposers of organic matter, whereas some of them create a lot of nuisances in the form of rotting of food crops, as well as secreting varieties of mycotoxins (ALTAF et al. 2017). *Penicillium* is also known to enhance plant growth and yield by providing available phosphorus and other plant-growth hormones such as IAA (indole 3- acetic acid) and gibberellins (ALTAF et al. 2017). Best known for the production of bioactive compounds (Lúcia & Enguita 2016), *Penicillium* became famous with the discovery of the antibiotic penicillin, which transformed medical approaches to treating bacterial pathogenic *Staphylococcus aureus* diseases (FLEMING, 1929,p. 245-250). Some *Penicillium* species are reported to have an endophytic lifestyle, which confers to them, the ability to protect their plant hosts against biotic stresses and promote plant growth (Waqas et al. 2015; LATIF et al. 2015). For instance, endophytic *P. citrinum* may play an important role in plant survival by enhancing nutrient uptake and producing growth-promoting metabolites such as gibberellins and auxins (KHAN et al. 2008)

With this background information, the current study has been carried out to evaluate de efficiency of the selected *B. amyloliquefaciens*, *E. nigrum*, *P. citrinum* in the rice blast disease suppression, (ii) in the stimulation of plant defense mechanisms, (iii) their interaction with rice

plants (iv) as well as rice cultivar BRSMG Caçula growth promotion and productivity, using microscopic, molecular, and pot assay under greenhouse conditions. In this study, the intention is to develop management strategies to minimize the use of chemical fungicides and fertilizers, and therefore their environmental impact by using these noted beneficial endophytic BCAs to improve the health of rice plants against blast disease and enhance plant growth and yields to meet food demand.

1.2. REVIEW OF LITERATURE

1.2.1. Rice plant and its importance

Rice is an annual herbaceous of tropical origin belonging to the *Gramineae* family and the genus *Oryza* (FAO 2016). It is cultivated in the tropics, subtropics, and warm temperate regions (Nguen 2013). This annual herbaceous plant, with erect culm (except for the floating varieties), is arranged in a tuft and bearing inflorescences in the form of a panicle (NGUEN 2013, p. 154). Among the cereals, rice occupies the largest proportion of the land and is probably the most diverse crop (KHUSH, 2005, p. 6). Rice, primarily for direct human consumption, remains an important staple food in Asia, Africa, Latin America, and the Caribbean (KOIZUMI & FURUHASHI, 2020). A major determinant of global rice consumption is the increasing demand from developing countries for rice in Asia and Africa. More than 90% of the world's rice is grown and consumed in Asia where 60% of the world population live (KHUSH 2005, p. 6). It is a grass, self-pollinating, which grows easily in tropical climates. However, it grows in a variety of strong environments but will grow faster and more vigorously in hot and humid conditions (KHUSH, 2005, p. 6). There are two main species of domestic and cultivated rice: *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice). This cultivated rice exists in thousands of varieties classified into two subspecies: Japonica and Indica (NGUEN, 2013, p. 154). The Japonica subspecies include tropical Japonica and temperate Japonica. However, the rice varieties are grown around the world largely belong to *O. sativa*. Many varieties of *O. sativa* cultivated in the world belong to two major subspecies: Indica, mainly long-grain rice characterized by wide adaptability to the different environments. And Japonica, round grain rice that is generally distinguished from Indica, by its high reactivity to

fertilizer applications (CALPE, 2006 cited by KOIZUMI; FURUHASHI, 2020). These two main types of rice as a common classification under *O. sativa* are traded in the world market (KOIZUMI; FURUHASHI, 2020). As stated by the Food and Agriculture Organization (FAO), the rice represents society, culture, politics, business, the scenic beauty of peoples within their communities, in short, rice is life. Africa is characterized by the paradox of low rice production (about 3.5% of world production) and a rapid increase in consumption (FAO, 2016). Nowadays, rice production is the main source of income and employment for more than 200 million households across the world and is the primary food for 2.5 to 3.5 billion people who are largely located in rapidly growing low-income countries (ASIBI et al. 2019). In 2002, rice provided more than 500 calories person^{-1} d^{-1} for over three billion people and a substantial amount of protein for 520 million people (MUTHAYYA et al. 2014). It is one of the most important cereals produced for food security and income by subsistence farmers (KARI & KORHONEN-KURKI 2013). Besides direct human consumption, rice grains are used to make alcohol, starch and derivatives, oils, pharmaceuticals, dietetic foods, etc. By-products such as bran and germs are used in livestock feed and the pharmaceutical industry. The bullets are used as building materials, fuel and ashes as fertilizers, in the manufacture of silica also. Rice straw is an important agro-residue used as feed for ruminants, also as a substrate for the production of edible mushrooms (SHEIKH et al. 2018).

1.2.2. Seedborne, airborne and leafspot pathogen *Pyricularia oryzae*

Belong to the Kingdom Fungi; Phylum of Ascomycota; Class Sordariomycetes; Order Magnaporthales; Family Pyriculariaceae (syn./Magnaporthaceae; Genus *Pyricularia* (syn./*Magnaporthe*; Species *Pyricularia oryzae* cavara (syn./*Magnaporthe oryzae* is an important plant pathogen (MCLAUGHLIN; SPATAFORA, 2015; ZHANG et al. 2016). Over 200 species of Magnaporthales have been described, of which about 50% are pathogens of cereals and wild monocotyledons (ZHANG et al. 2018). It is best known as the most destructive disease of rice and wheat (ZHANG et al. 2018). *P. oryzae* was the first designation of the rice blast pathogen in 1892 (AGARWAL et al., 1994). Thus, many other *Pyricularia*-like isolates that were recovered from blast lesions on barley (*Hordeum vulgare*), millets (*Eleusine coracana*, *Pennisetum glaucum*,

Setaria italica), oats (*Avena sativa*), perennial ryegrass (*Lolium perenne*), wheat (*Triticum aestivum*), and more than 50 other grass species were also classified under the same species (Ceresini et *al.* 2016; COUCH et al. 2005; MURAKAMI et *al*. 2000) (Table 1). According to Sesma; Osbourn (2004), *P. oryzae* life cycle is the other face is the root infection. The rice blast fungus can be found referenced in the literature under several names as *P. oryzae* was used to refer to the asexual stages of the pathogen as it was found in the field (EBBOLE, 2007, p. 437-456). The sexual stage was named *Magnaporthe grisea* until it was shown by phylogenetic analysis (CERESINI et al. 2016). This pathogen has evolved different strategies to overcome the various barriers that, they encounter during infection of their hosts (Gladieux et al. 2018). It has been shown that *P. oryzae* invariably causes the death of seedlings through seed and transmission of the primary leaf pathogen to 30% (CASTROAGUDÍN et al. 2016). These same authors had found that *P. oryzae* can be transmitted from seed to seedling, and the blast disease can be introduced into an uninfected area through the seed (MARTINS et al. 2004). According to Agarwal et al. (1994), *Pyricularia* conidia are found on the seed surface and resting mycelium in the tissues of the embryo, endosperm, layers of bran and lemmas, and also between lemmas and seed. The spread of this airborne pathogen is mainly occurred by wind and seed. According to Suzuki et al. (1969), the outbreak of blast in 1967 in Japan was attributed to seed infection. It has been reported that moderate infection in the field could result in 7 to 18% of seed infections (AGARWAL et al. 1994).

Table 1. Grasses and cereals parasitized by species of *Pyricularia*

Scientific name	Common name	Scientific name	Common name
Agropyron repens	Quackgrass	*Holcus lanatus*	common velvet grass
Agrostis palustris	Creeping bentgrass	*Hordeum vulgare*	common barley
A. tenuis	Colonial bentgrass	*Hystrix patula*	Eastern bottlebrush grass
Alopecurus pratensis	Meadow foxtail	*Leersia hexandra*	Southern cutgrass
Andropogon sp.	Bluestem	*Hierochloe odorata*	Vanilla grass
Anthoxanthum odoratum	Sweet vernalgrass	*L. japonica*	
Arundo donax L.	Giant reed	*L. oryzoides*	
Avena byzantina	Oat	*Lolium italicum*	Italian ryegrass
A. sterilis	Oat	*L. multiflorum*	
A. sativa	Oat	*L. perenne*	perennial ryegrass
Brachiaria mutica	para grass	*Muhlenbergia* sp.	Muhly
Bromus cathartics	Rescue grass	*Musa sapientum*	French plantain
B. inermis	smooth brome	*Oplismenus undulatifolius*	Wavy leaf basket grass
B. sitchensis	Alaska brome	*Oryza longistaminata*	Long stamen rice or red rice
Canna indica	Indian shot	*Oryza sativa* L.	Commun rice
Chikushichloa aquatica		*Panicum miliaceum*	
Costus speciosus	Canereed	*P. ramosum*	signalgrass
Curcuma aromatica	Curcuma	*P. repens* L.	torpedo grass
Cynodon dactylon L.	Bermudagrass	*Pennisetum typhoides*	Pearl millet
Cyperus rotundus	nutgrass	*Phalaris arundinacea*	Reed canarygrass
C. compressus L.	portland flatsedge	*P. canariensis*	canarygrass
Dactylis glomerata	Orchardgrass	*Phleum pretense*	timothy
D. sanguinalis	hairy crabgrass	*Poa annua* L.	annual bluegrass
Echinochloa crus-galli	Barnyardgrass	*P. trivialis*	rough bluegrass
Eleusine indica	Indian goosegrass	*Saccharum officinarum*	sugarcane
Eragrostis sp.	Purple lovegrass	*Secale cereale*	Cereal rye
Eremochloa ophiuroides	Centipede grass	*Setaria italica*	foxtail bristlegras
Eriochloa villosa	Cupgrass	*S. viridis*	green bristlegrass
Festuca altaica	Altai fescue	*Sorghum vulgare*	grain sorghum
F. arundinacea	Tall fescue	*Stenotaphrum secundatum*	St. Augustine grass
F. elatior	meadow ryegrass	*Triticum aestivum*	common wheat
F. rubra	red fescue	*Zea mays* L.	Corn
Fluminea sp.		*Zingiber mioga*	Mioga ginger
Glyceria leptolepis		*Z. officinale*	garden ginger
Hierochloe odorata	Vanilla grass	*Zizania latifolia*	Manchurian wild rice

Source: Natural Resource Conservation Service, USDA, 2004 (USDA 2004) amended.

1.2.2.1. Symptoms and disease development

Rice blast affects the leaves, on which it causes lozenge-shaped white to grey or reddish-brown lesions with reddish to brown borders (AGARWAL et al. 1994). The lesions may enlarge, coalesce, and kill entire leaves. These lozenge-shaped or diamond-shaped lesions on the leaves are a key characteristic for the quick identification of the disease in the field (Sesma & Osbourn 2004). The blast also affects the leaf collar, which it may kill, and the stem nodes and occasionally the internodes, which at heading may result in the production of white panicles or breakage of the stem at the infected node (AGRIOS, 2005, p. 922). The blast lesions on leaves are called leaf blast, those on leaf collars (collar blast), others on the stem, stem nodes, neck nodes (neck rot), panicles (panicle blast), seeds and even the roots, which vary in color and shape depending on cultivars resistance, environmental conditions, and plant age (ASIBI et al. 2019; SESMA; OSBOURN 2004; ZHANG et al. 2016;) (Fig.1 A-D).

This seed-borne and foliar pathogen *Pyricularia* over seasons as mycelium and conidia on diseased rice straw, seed, and on weed hosts such as *Oryza longitaminata* (RAVELOSON et al. 2013). In the tropics, conidia are present in the air throughout the year (AGRIOS, 2005, p. 922). The dissemination of the fungus during an epidemic occurs by aerial dispersal of conidia (EBBOLE, 2007, p. 437-456). The fungus produces and releases conidia when the environmental factors relating to the host-parasite pair are favorable, during periods of high relative humidity (FANAMBINANA et al. 2010). Apart from the propagation of the pathogen at short range becoming airborne and on landing on rice plant, they adhere strongly through sticky mucilage they produce at their tip (FANAMBINANA et al. 2010). The seed infected is at the origin of the dispersal of the pathogen at a great distance allowing it to invade other agroecosystems (RAVELOSON et al. 2013; Ceresini et al. 2016). Also, Sesma; Osbourn (2004) reported that the systemic rice plant invasion by *M. grisea* through the roots may contribute to the establishment of rice blast disease in the field. This phase of the infective process occurs between 12 and 24 h after the contact between the conidia and the rice plant (ARRIEL-ELIAS et al. 2019). In addition, the production and accumulation of melanin in the appressorium cell wall are necessary for successful penetration. Rice seedlings, young leaf and stem tissues are more susceptible than older plants and

tissues (AGARWAL et al. 1994). Following the penetration, the fungus ramifies intra- and intercellularly in susceptible host tissue before lesion formation initiates, with host cell death starting 4 to 5 days after infection (AGRIOS 2005, p. 922; EBBOLE 2007, p. 437-456) (Fig.2). Movement into the next cell occurs specifically at clusters of plasmodesmata (Ebbole 2007, p. 437-456). In wet weather or high relative humidity, new conidia are produced and released within hours from the appearance of the lesions and this continues for several days, with most conidia being released between midnight and sunrise (AGRIOS 2005, p. 922). Environmental conditions favoring sporulation and lesion development that occur during the night include extended periods of leaf dampness (relative humidity) is between 92% to 100%, and air temperature is between 22°C to 28°C (ASIBI et al. 2019; KANKANALA et al. 2007). Rice blast is favored by high nitrogen fertilization, prolonged leaf wetness, drought stress (AGRIOS 2005, p. 922; ASIBI et al. 2019), the use of intensive agronomic practices as well as rainfall and high humidity (ALI; NADARAJAH, 2014). Freitas et al. (2010) suggested that the leaf pathogens are generally favored by higher nitrogen levels, which promotes greater development and therefore, softer more succulent tissues. FANAMBINANA et al. (2010) reported that the use of a high dose of nitrogen decreases hemicellulose and lignin in the cell wall of the rice plant, thereby weakening its resistance mechanism and accentuating the development of the disease. In addition, high nitrogen significantly reduces the content of phenolic compounds which are toxic to the pathogen (Roger, 1990 cited by FANAMBINANA et al. 2010). According to these same authors, the level of silica in the epidermal cells plays a mechanical role in the resistance to the penetration of pathogens. This silica is reduced by the nitrogenous excess while increasing the vulnerability of the plant to diseases. The increased rice leaf blast had been also attributed to the increased canopy density (KÜRSCHNER et al. 1992).

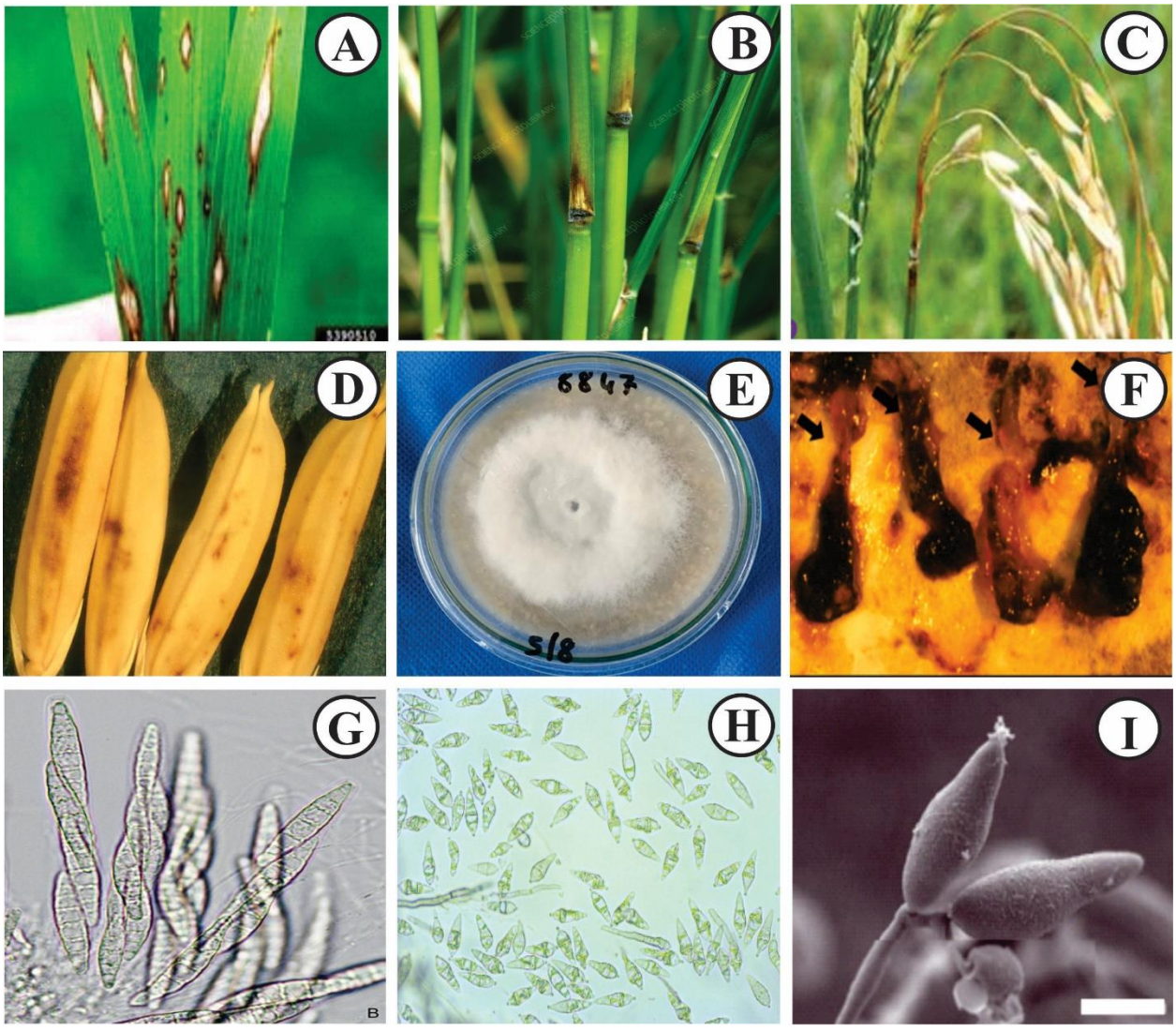

Figure 1. Rice blast disease symptoms and *Pyricularia oryzae* morphology, fruiting body, and spore. Rice leaf blast symptoms (Donald Groth, Louisiana State University AgCenter, Bugwood.org) (A) by copyright permission. Nodes blast (B); neck nodes (neck rot) (C) (NGUEN 2013, p. 154); panicle blast (D) (AGARWAL et al. 1994). Mycelium of *P. oryzae* was produced on a Petri dish containing AO medium after 15 days of culture on an oat-meal agar medium at 22°C in photoperiod light and darkness (E). Light microscope image of perithecia in the culture medium, showing long necks (F) (Moreira et al. 2015). Asci of *P. oryzae* (G) (KATO ; YAMAGUCHI, 1982). *P. oryzae* conidium observed on optical microscopy (H). Scanning electron micrograph of *Pyricularia oryzae* conidium on conidiophore (I) (WILSON; TALBOT, 2009).

1.2.2.2. Rice blast disease cycle

Rice blast, a polycyclic disease is caused by a filamentous ascomycete fungus and spread by asexual spores (conidia) that infect all tissues of rice plants (WILSON; TALBOT 2009). The aerial conidiophores produce a sympodial arrangement of conidia and these spores from the inoculum for secondary infection cycles (EBBOLE 2007, p. 437-456). Conidia in water germinate by producing a single germ tube within 30 min after contact with leaf surface (HOWARD; VALENT, 1996) (Fig.2). A single germ tube emerges from the apical and/or basal cell of three-celled conidia. Germ tubes developing on surfaces appear to be associated with an extracellular matrix material (HOWARD; VALENT, 1996). Conidia contain a spore tip mucilage that is released upon hydration and forms a strong adhesive to irreversibly attach the conidium to the surface before germination (EBBOLE 2007, p. 437-456). This spore tip mucilage is thought to be composed of mannose containing carbohydrates and glycoproteins (EBBOLE, 2007, p. 437-456). Rice blast disease is triggered by a specialized infectious cell called appressoria which swells and differentiates from the ends of a polarized germ tube by developing turgid pressure (Egan et al. 2007). Mechanically, it breaks the rice cuticle before invading the underlying cells of the epidermis (FANAMBINANA et al. 2010; EGAN et al. 2007;). Melanin deposition in the cell wall of the appressorium is essential for maintaining turgor pressure on rice tissues (HOWARD; VALENT, 1996; OH et al. 2008). According to Sesma; Osbourn (2004), the melanized appressoria is not observed on the surface of rice roots when it is infected by *M. oryzae*, which is like hyphopodia at infection sites, often associated with infection pegs. *P. oryzae* is a hemibiotrophic pathogen that assumes a biotrophic lifestyle during the initial stages of infection but later adopts a necrotrophic lifestyle before completing their life cycles (ZHOU, 2016, p. 5). Fungal growth within rice cells causes the death of the infected tissues and necrotic lesions within 3 to 5 days (AGRIOS, 2005, p. 922) (Fig.2). The pathogen survives in the residue of host plants tissues and the cycle repeats(RAVELOSON et al. 2013). Under favorable conditions, it can produce one cycle per week, with a single lesion producing hundreds of spores each night for more than 20 days (KATO et al. 1982).

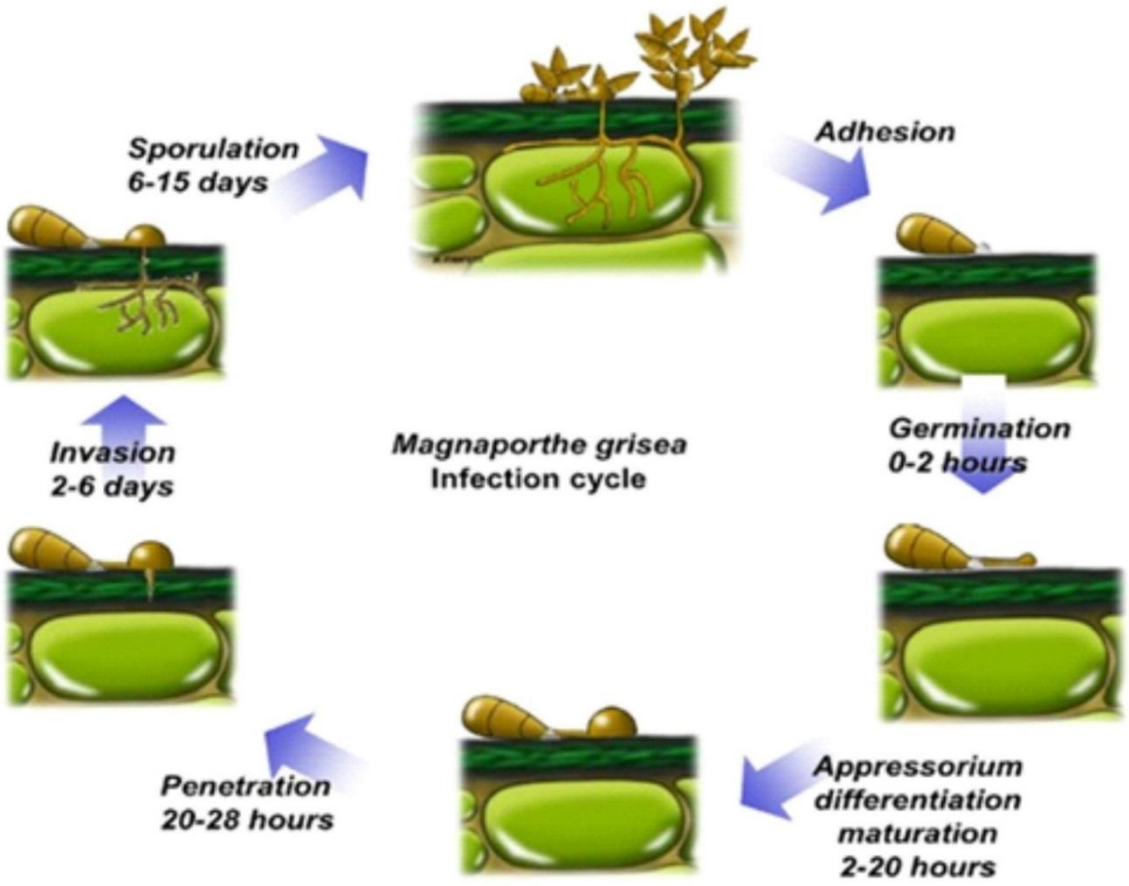

Figure 2. *Pyricularia oryzae* infection cycle according to Ribot et al. (2008)

1.2.2.3. Secondary metabolites of *Pyricularia oryzae* and host colonization

Several microbial species have evolved the ability to infect, grow, develop, and complete their life cycle, but the outcomes are diverse. Pathogens enter and colonize plants in different ways, feed on plant cells, adversely alter plant metabolism, cause a range of detrimental disease symptoms, and lower yields (BUCHANAN et al. 2015). Some pathogens penetrate surface layers directly using mechanical pressure or enzymatic attack, some of them pass-through natural openings, such as stomata or lenticels, and others only enter through wounded tissue (BUCHANAN et al. 2015). Once inside the plant, one of three main attack strategies called pathogenesis is deployed: necrotrophy, where the plant cells are killed in advance of infection; biotrophy, where the plant

cells remain alive throughout infection; and hemibiotrophic, where the pathogen initially keeps cells alive but at later stages of the infection, kill them (BUCHANAN et al. 2015). According to Chagas et al. (2018), the secondary metabolites with antimicrobial activity that are constitutively produced by plants are called phytoanticipins. The inducible compounds with antimicrobial activity that are produced by plants during pathogen infections are known as phytoalexins (CHAGAS et al. 2018). Indeed, *Pyricularia*, which causes the most serious damage to rice, and recently to the wheat, is a hemibiotrophic fungus (NISHIMURA et al. 2016). To cause plant diseases, pathogen secretes effectors proteins into host tissue to suppress immunity and support pathogen growth. The rice blast fungus *P. oryzae* has evolved distinct secretion systems (amount of two) during plant infection facilitating tissue invasion (GIRALDO et al. 2013). *Pyricularia* then invades rice tissue using specialized filamentous invasive hyphae (IH), which successively occupy living rice cells and colonize tissue extensively before the appearance of disease symptoms (BUCHANAN et al. 2015) (Fig.3). A structure called biotrophic interfacial complex (BIC) is a plant-derived, membrane-rich structure that facilitates translocation of some effectors proteins into the host cell cytoplasm (GIRALDO et al. 2013; MENTLAK et al. 2012). It is focally formed at the periphery of the invasive hyphae (NISHIMURA et al. 2016). These effectors reach the rice cytoplasm, then move via plasmodesmata into uninvaded neighboring host cells, presumably preparing them before the invasion (BUCHANAN et al. 2015). Cytoplasmic effectors show preferential accumulation in the BIC, which is first located in front of the growing primary hyphal tips, and then remains behind beside the first-differentiated bulbous IH cell (GIRALDO et al. 2013). Inside the cell lumen, the invasive bulbous hyphae and intensive division (IH) are surrounded by a membrane of plant origin which separates the IH and the cytoplasm from the host, characteristic of biotrophy (KANKANALA et al. 2007) (Fig.3). Fungal progression to the next cell occurs in the vicinity of pit fields without causing visible damage to the cell wall and crossing the cell wall at plasmodesmata (KANKANALA et al. 2007). The invasion of neighboring cells coincides with the loss of viability of the previously infected cell, and the start of necrotrophic growth leading to the appearance of lesions 74 to 96 h after inoculation in laboratory conditions (WILSON; TALBOT, 2009). During the biotrophic invasion, *P. oryzae* expresses many low-molecular-weight biotrophy-associated secreted (Bas) proteins, including effectors proteins, and

these proteins possess classical signal peptides, which facilitate delivery into the endoplasmic reticulum (ER) (MOSQUERA et al. 2009; VALENT; KHANG, 2010). Effectors are defined as molecules or compounds used by microorganisms to suppress, manipulate or evade plant responses (FORUM LETTERS, 2016, p. 805–813). Four candidates have been confirmed to be fungal biotrophy-associated secreted (BAS) proteins (MOSQUERA et al. 2009). Fluorescently labelled BAS proteins were secreted into rice cells in distinct patterns of incompatible interactions. BAS1 and BAS2 proteins are accumulated in biotrophic interfacial complexes along with known avirulence effectors, BAS3 showed additional localization near cell wall crossing points, and BAS4 uniformly outlined growing IH (MOSQUERA et al. 2009).

Liu & Wang (2016) reported that nowadays, 21 Avr effector genes (ex: AvrPib, AvrPi-ta for *P. oryzae* and AvrXa3 for *Xoo*), have been cloned in rice pathogens, including 13 from *M. oryzae*. For Sakulkoo et al. (2018) a single fungal mitogen-activated protein kinase (Pmk1) regulates the expression of secreted fungal effectors proteins implicated in the suppression of host immune defenses. Pmk1 also prevent reactive oxygen species (ROS) generation and excessive callose deposition at plasmodesmata and controls the hyphal constriction required for fungal growth from one cell to another cell, enabling host tissue colonization and blast disease development (SAKULKOO et al. 2018). The genome of *P. oryzae* contains three mitogen-activated protein kinase (Mapk cascades that regulate appressorium development, penetration peg formation and adaptation to hyper-osmotic stress for its virulence (DEAN et al. 2005).

The rice blast fungal secondary metabolites (pyriculol and pyriculariol) appear to induce lesion formation on rice leaves but are dispensable for the pathogenicity of *P. oryzae* (JACOB et al. 2019). It is estimated that 23 polyketide synthases (a large class of secondary metabolites) are present in the genome sequence of *P. oryzae*, along with 6 nonribosomal peptide synthetases and 8 nonribosomal peptide synthetase/polyketide synthetase hybrid genes (DEAN et al. 2005). One of the best-characterized metabolites is tenuazonic acid, a photosystem II inhibitor that is thought to involve in the condensation of isoleucine, and its synthesis could involve the activity of peptide and a polyketide to bind the substrates for condensation (EBBOLE, 2007, p. 437-456).

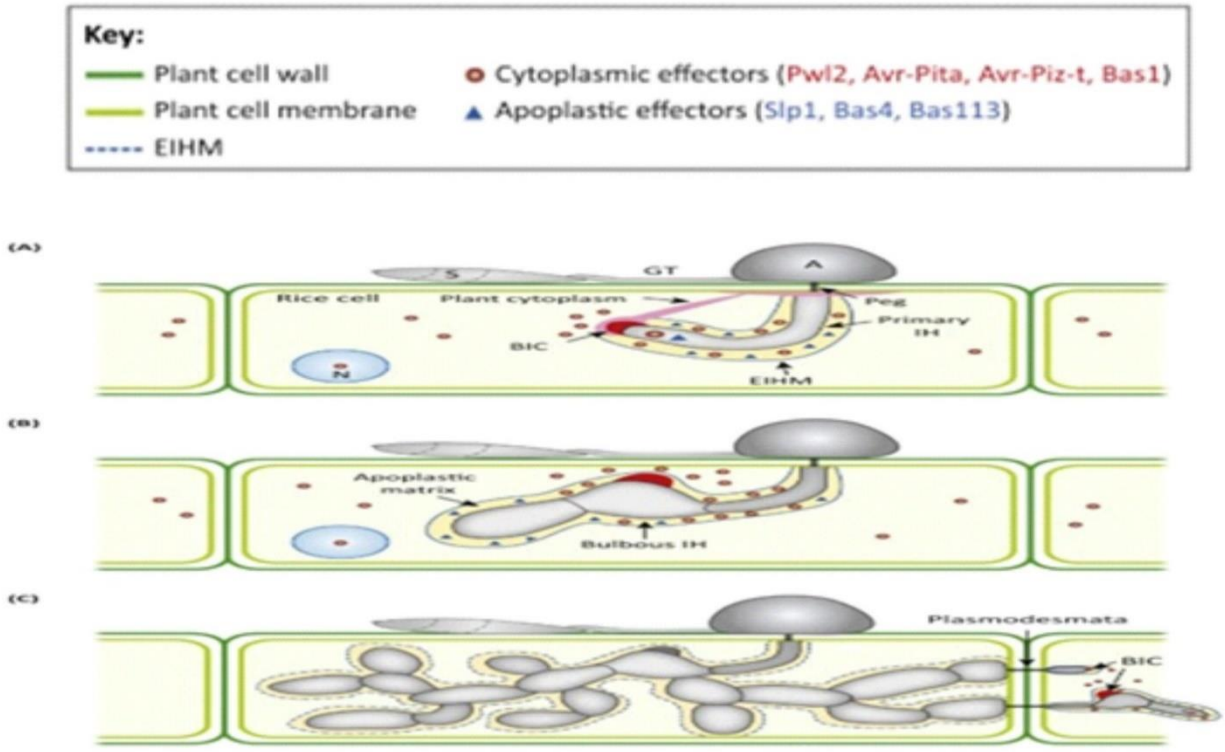

Figure 3. Development of the biotrophic interfacial complex (BIC) and effectors trafficking during the biotrophic invasion of rice cells (a–c) according to Fernandez & Orth (2018). On the rice surface, a conidium germinates and produces a pressurized appressorium to mechanically breach the host cuticle and epidermal cell wall (GIRALDO et al. 2013; VALENT & KHANG, 2010). Schematic representation of the differentiation of a filamentous primary invasive hypha (a, 22–25 hpi); into a pseudo-hyphal-like bulbous invasive hypha (b, 26–30 hpi.); in a first-invaded rice cell, the differentiation occurs for each new hypha invading a living neighbor cell (c, 36–40 hpi).

1.2.2. Rice plant defense mechanisms against *Pyricularia oryzae*

Each plant species is frequently affected by hundreds of different kinds of fungi, bacteria, mollicutes, viruses, insects, and nematodes (LIU; WANG 2016; AGRIOS, 2004, p. 922). Understanding defense mechanisms in rice are essential, given its great economic importance and the devastating nature of rice blast disease. In general, plants defend themselves against pathogens by a combination of weapons from two arsenals: first, the structural mechanisms (cuticle, trichomes, wax, papillae, tyloses, cork layers) that act as physical barriers and inhibit the pathogen from gaining entrance and spreading through the plant (BUCHANAN et al. 2015). Secondly, the

biochemical reactions (phenols, alkaloids, phytotoxins, phytoalexins, PRs, R genes (HR) (BUCHANAN et al. 2015). This last one takes place in the cells and plant tissues and produce substances that are toxic to the pathogen or create conditions that inhibit the growth of the pathogen in the plant (BUCHANAN et al. 2015). The combinations of structural characteristics and biochemical reactions employed in plants defense are different according to host-pathogen systems.

According to the standard zigzag model, rice plants recognize *P. oryzae* attack through plant cell surface pattern recognition receptors (PRRs), host plants can specifically recognize pathogen-associated molecule patterns (PAMPs), which include microbial secondary metabolites (CHAGAS et al. 2018) (Fig.4). PRRs are represented by transmembrane receptor-like kinases (RLKs), which typically contain extracellular leucine-rich repeats, an intracellular kinase domain, and receptor-like proteins (RLPs) (BUCHANAN et al. 2015). Then it activates defense response by cell wall modification, callose deposition, and via expression of defense-related (PR) proteins in host cells, which is called PAMP-triggered immunity (PTI) (Ning et al. 2020). To circumvent PTI, fungal, bacterial, viral, and nematode pathogens evolve effector proteins that suppress host defenses leading to effectors-triggered susceptibility (ETS) (LIU; WANG, 2016). *P. oryzae* can secrete certain effectors to inhibit PTI and can break resistance responses in several cases (MENTLAK et al. 2012). Quickly, rice has acquired more specific resistance proteins that directly or indirectly recognize pathogen effectors proteins (NING et al. 2020). Findings have shown that conserved PAMPs such as peptidoglycan, lipopolysaccharide, and fungal chitin can be sensed by rice cells and trigger innate immunity (CHEN; RONALD, 2011). For instance, in rice, the chitin elicitor binding protein (CEBiP) recognizes chitin oligosaccharides released from the cell walls of pathogens, and *M. oryzae* overcomes this first line of plant defense by secreting Secreted LysM Protein1 (Slp1) effector protein during the invasion of new rice cells (MENTLAK et al. 2012). Slp1 is required by *M. oryzae* for complete virulence and exerts significant tissue invasion and lesion expansion. In contrast, gene silencing of CEBiP in rice allows *M. oryzae* to induce lesions in the absence of Slp1 (MENTLAK et al. 2012). According to this same source, Slp1 sequesters the oligosaccharides of chitin to prevent the immunity triggered by PAMP in rice, thus facilitating the rapid spread of the fungus in the tissues. The recognition mechanism normally activates the

second branch, which mostly acts within the cell, uses highly polymorphic resistance (R) proteins in rice, that respond to pathogen effectors leading to a rapid and robust effector-triggered immunity (ETI) (CHAGAS et al. 2018). This fact results in the production of ion (Ca2+, K+ and H+) currents, superoxide, nitric oxide, and programmed cell death at the site of invasion (Liu & Wang 2016; CHAGAS et al. 2018). ETI is a highly specialized disease resistance mechanism in the host, which is activated in the gene-for-gene model upon recognition by an R (resistance) protein of the corresponding effector protein of *M. oryzae* (avirulence genes) (NING et al. 2020). Many effector encoding genes are called avirulence (Avr) genes because they are recognized by a cognate resistance gene. To date, more than 40 AVR genes have been identified in *P. oryzae*, while 12 of them have been cloned (NING et al. 2020; WANG et al. 2017). For example, *P. oryzae* carries the avirulence gene Avr-Pi-ta effective on rice cultivars carrying the resistance gene Pi-ta. This Pi-ta encodes a cytoplasmic protein that contains an NBS domain and a leucine-rich carboxyl terminus (AGRIOS, 2005, p. 922). For example, the rice genome contains about 480 nucleotide-binding sites leucine-rich repeat (NLR) genes, while the human genome has only about 10 (ZHOU et al. 2004). Resistance to rice blast is usually mediated by R genes, most of which are nucleotide-binding site leucine-rich repeat (NLR) genes (WANG et al. 2019). Also, the rice hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are important regulators of immune responses and they play important roles in plant immunity (JIANG et al., (2010). SA is usually considered to regulate immunity against biotrophic pathogens, whereas JA and ET are believed to be involved in resistance to necrotrophic and insect pests (BUCHANAN et al. 2015). One of the earliest strategies of the plant defense response is the production of reactive oxygen species (ROS), hydrogen peroxide (H2O2), the hydroxyl free radical (OH^-) and superoxide anion (O_2^-) (WOJTASZEK, 1997, p. 681-692). Its resistant pathogens attack directly, strengthen plant cell walls through the oxidative cross-linking of cell wall structural proteins (BUCHANAN et al. 2015) and have been proposed to drive programmed cell death (TORRES et al. 2005). Rice plants can produce some antioxidant defense enzymes such as Superoxide dismutase (SOD), Peroxidase (POX), Polyphenol oxidase (PPO), and Phenylalanine ammonia-lyase (PAL) against pathogens through pathogen attack or by antagonist elicitation (RAIS et al. 2017).

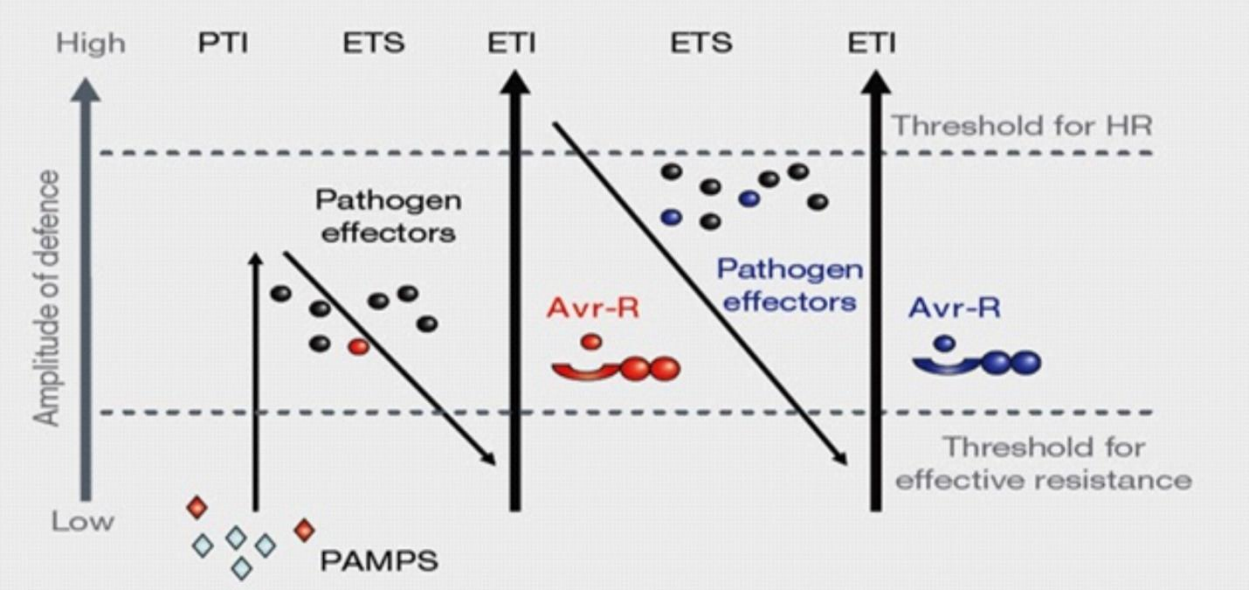

Figure 4. The general model of plant-pathogen interactions (Zig-Zag-Zig model) according to Buchanan et al. (2015).

1.2.3. *Pyricularia* species resistance to fungicides

Rice blast, caused by the fungus *P. oryzae* importance can be measured using several different parameters, particularly its geographical distribution is wide (present in all rice-growing areas of the world) (BALLINI et al. 2008). The costs of fungicides were estimated at 160 million Euros in Japan in 2000 and 2.4 to 8.9 million hectares treated in China from 1980 to 1990 (BALLINI et al. 2008). Management of rice blast has relied on treatment with fungicides because resistant cultivars become vulnerable within a few years after release (BALLINI et al. 2008). Nguyen et al. (2016) reported that cultivars with durable are scarce in the field because of the pathogen's ability to evolve to overcome resistance genes. Also, the continuous use of chemical inputs causes several negative effects such as the development of pathogen resistance to the applied agents, and their nontarget environmental impacts (COMPANT et al. 2005), in addition to the pathogens mutant selection strains (Nguyen & al. 2016). According to Dorigan et al. (2019), several species of *Pyricularia* such as *P. graminis-tritici* (Pygt), are resistant to triazole fungicides including tebuconazole, and epoxiconazole with the presence of the CYP51A mutation gene, which encodes the target enzyme 14-alpha- demethylase. In Japan, a fungicide carpropamid on scytalone

dehydratase (SDH) was introduced in 1998 as an effective and specific fungicide in the control of rice blast (SUZUKI et al. 2010). However, after three years, the failure of carpropamid to control blast was reported in 2001, and the presence of isolates resistant to carpropamid was confirmed there (TAKAGAKI et al. 2004). This resistance of *P. oryzae* to carpropamid was associated with a single point mutation in the SDH gene which resulted in the replacement of valine by methionine at amino acid position 75 (V75M) (TAKAGAKI et al. 2004). Also, the strains of *P. oryzae* resistant to the organophosphorus fungicide pyrazophos have been observed (WAARD; VAN NISTELROOY, 1980). In addition, Castroagudín et al. (2015) reported that *M. oryzae* resistance to strobilurin-based fungicides (outer quinone [QoI] inhibitors) had been observed in the wheat fields and poaceous hosts in central and southern Brazil. The use of chemicals fungicide leads to negative environmental effects such as toxicity, ecotoxicity, and the appearance of resistant strains of the pathogen (RAVELOSON et al., 2013). According to Son et al. (2018), plant protection products (PPP) can get satisfying results in agricultural production, but their use is risky to human health, the environment and non-target organisms. A synthesis of available data suggests that the over-application of pesticides contribute to the higher risk in vegetable production of toxins and highly concentrated PPPs without appropriate protective equipment (SON et al. 2018).

1.2.4. *Pyricularia* overcome host resistance genes

Breeding for disease resistance remains the top-most cost-effective, labor-saving method, and an environmentally friendly strategy (CHUMA et al. 2011). The resistance of cultivars conferred by major genes has been widely used for the selection of many crop species (BUCHANAN et al. 2015). However, the new resistant varieties were often rendered ineffective a few years after their release in farmers' fields (NGUYEN et al. 2016; RAVELOSON; MATHILDE 2012). This break-in resistance was caused by a rapid adaptation of the pathogen, that is, the evolution of new races which overcome the resistance genes introduced (BUCHANAN et al. 2015; NING et al. 2020). The large-scale cultivation of a new cultivar with a major resistance gene and its failure by a new pathogen race phenomenon has been called the boom-and-bust cycle (BUCHANAN et al. 2015). The rapid evolution of the new cultivar is attributed to the rapid loss of function of virulence

effectors genes which corresponds to the resistance genes in a gene-for-gene manner (CHUMA et al. 2011). Fungal populations often recover the avirulence genes after varieties containing the corresponding resistance genes have been removed from the field. A simple explanation for this recovery phenomenon is that isolates carrying the avirulence gene had survived as a minor race in the field during the predominant cultivation of a resistant cultivar carrying its corresponding resistance gene (MENTLAK et al. 2012). Then, when we stopped cultivating this resistant cultivar, their population increased again due to the contribution of the avirulence gene. Or, isolates carrying the virulence gene migrated to regions where the resistance gene had not been deployed (MENTLAK et al. 2012). Sexual recombination would allow isolates that have been deleted for an avirulence gene to recover that gene, but pathogens such as *P. oryzae* are predominantly asexual in the field. The specificity of *P. oryzae* and race-cultivar in *Oryza* isolates is governed by gene-for-gene interactions (SILUÉ et al. 1992). Three cloned avirulence genes are involved in host species specificity: PWL1, PWL2 and AVR1-CO39. The cloned avirulence genes involved in the specificity of the rice cultivar are AVR-Pita, ACE1, AvrPiz-t, AVR-Pia, AVR-Pii and AVR-Pik/km/kp (Dean et al. 2005). It has been suggested that suppression of AVR-Pita is a common mechanism to overcome resistance in the field, although point mutations and insertions of transposable elements also lead to virulence (EBBOLE, 2007, p. 437-456). Among the identified and cloned resistance genes, Pi1, Pi5, Pi33, Pi54, Piz, Piz-t, Pi2, Pi9, Pi40 and Pigm are broad-spectrum resistance genes to leaf blast (CHUMA et al. 2011). Pi1 in the LAC23 cultivar from West Africa shows resistance to 98% of 792 *M. oryzae* isolates in China (NING et al. 2020). Mutation or deletion in AVR genes permits the pathogen to evade detection by the plant, potentially allowing infection (COUCH et al. 2005). The rice Xa21 gene confers broad-spectrum resistance to diverse strains of *Xanthomonas oryzae* pv. *oryzae* (Xoo), which encodes a receptor kinase carrying extracellular leucine-rich repeats (LRRs) as well as transmembrane (TM), and intracellular non-RD (arginine - aspartate) kinase domains (CHEN; RONALD 2011). Chuma et al. (2011) reported that despite the use of resistant cultivars, blast epidemics can still occur, due to a lapse in host resistance and the emergence of new virulent pathotypes.

1.2.5. Biological control agents (BCAs) and their mode of actions

Biocontrol is based on the exploitation by humans for his benefit the natural relationship between an animal, insect, and or pathogen of crop and another organism, most often a parasite, a predator, or a pathogenic agent of the first, which kills it in nourishing (Jourdheuil and Fraval 1991). In other words, biological control is the control of a microorganism through another microorganism by reducing the sum of the inoculum or of the activities that determine the disease, caused by a pathogen, carried out by one or more organisms other than a man (CONRADO; SANTOS, 2017). Microorganisms involved in the biological control are fungi, bacteria and viruses. Biological control agents (BCAs) are applied to control plant pathogens, where they act via a range of modes of action (KÖHL et al. 2019). Some of them could control disease (primary effect) but have demonstrated stimulation of plant growth (secondary effect) in the absence of a pathogen (AVIS et al. 2008). For instance, the disease suppression by *B. subtilis* is the net result of multiple mechanisms, including plant growth promotion (PGP), antibiosis, competition for space and nutrients, lysis of pathogen hyphae, and induced systemic resistance (ISR) (WANG et al. 2018). Most of BCAs bacteria exhibit several mechanisms that may affect the disease triangle directly, indirectly, or synergistically (WANG et al. 2018). Fungi or bacteria could improve plant growth and development directly or indirectly. The direct mechanisms may prevent the growth or activity of the pathogens through competition for space and nutrients, antibiosis, production of hydrolytic enzymes, inhibition of pathogen-produced enzymes or toxins, and through ISR defense mechanisms (CONRADO; SANTOS 2017). Whereas, the indirect mechanisms may involve nitrogen fixation, production of regulators such as auxins, cytokinins and gibberellins, and suppression of stress ethylene synthesis by 1-aminocyclopropane- 1-carboxylate (ACC) deaminase activity. The chemical indices are important in microbe-microbe interactions (CHAGAS et al. 2018). The positive effects of many bacteria on plants are mediated by a range of mechanisms, including improvement of mineral nutrition, plant tolerance to biotic and abiotic stresses through the production of several molecules such as bacteriocin, surfactin, QS (HAO et al. 2016; KLOEPPER et al. 1999). According to Chagas et al. (2018), microbial endophytes present themselves as a producer of stimulators of many bioactive metabolites such as flavonoids, peptides,

alkaloids, steroids, phenolics, terpenoids, flagella, QS, siderophore, auxin, cytokinin, gibberellins, etc. ISR is generally triggered by endophytes when the pathogens attack the host plants far from their point of colonization, which depends mainly on the involvement of JA and ET signaling (DERKSEN et al. 2013). For instance, necrotrophic plant pathogenic bacteria, fungi and oomycetes kill and subsequently invade tissues of host plants and utilize the available nutrients as primary colonizers of these killed tissues (KÖHL et al. 2019). Non-pathogenic saprophytic endophytes such as *E. nigrum* may have a competitive advantage and may play an important role in competitive substrate colonization of leaf lesions caused by necrotrophic pathogens (SENA et al. 2013). Besides these mechanisms of phytopathogen control, numerous plant growth-promoting rhizobacteria (PGPR) used for their bio fertilization and biocontrol activities play an important role in plant growth via various mechanisms such as nitrogen fixation, growth hormone production, solubilization of phosphate, production of siderophores, production of hydrolytic enzymes, antagonistic activities against fungal pathogens (TAILOR; JOSHI, 2014).

1.2.6. Antifungal properties of *Bacillus* species

Bacillus is a genus of gram-positive bacteria that produce endospores and have a unique ability to replicate rapidly, is resistant to adverse environmental conditions as well as has a broad spectrum of biocontrol ability (SHAFI et al. 2017). Several species of *Bacillus* are found naturally and are therefore considered as plant growth-promoting rhizobacteria (PGPR) isolated from the rhizosphere of several cultivated plants (SARANRAJ, 2014, p. 1265-1277). Most of the species from the *Bacillus* genus are considered safe microorganisms and they possess remarkable abilities to synthesize many substances that have been successfully used in agriculture (STEIN, 2005, p. 845-857). Secondary metabolites produced by several species of *Bacillus* have been found to show antibacterial or antifungal activity against several phytopathogens (ARGUELLES-ARIAS et al. 2009). For Beri et al., (2012), *Bacillus* spp. growth fast in liquid culture and form resistant spores with high thermal tolerance, which makes these bacteria ideal candidates for use as biocontrol agents. Some studies suggested that the volatile compounds produced by *B. subtilis* play an important role in plant growth promotion and the activation of a plant defense mechanism by

triggering the induced systemic resistance (ISR) in plants (COMPANT et al. 2005; SHAFI et al. 2017). Members of *Bacillus* spp. are generally bacteria living in the soil or exist in the form of epiphytes or endophytes in the spermosphere or rhizosphere having an antagonistic and inhibiting activity (SARANRAJ, 2014, p. 1265-1277; SHAFI et al. 2017). They synthesize various types of lipopeptides based on secondary metabolites with specific activities against plant pathogens, which give them unique importance in agriculture (SHAFI et al. 2017). *Bacillus* species synthesize many potent amphiphilic (hydrophilic and lipophilic) and surfactant lipopeptides comprising bacillomycins, iturins and mycosubtilin (GONG et al. 2015). According to Saranraj (2014, p. 1265-1277), *Bacillus* is one of the abundant microorganisms in the rhizosphere, having the PGPR activity, resulting in the synthesis of auxin, cytokinin and gibberellins metabolites. *Bacillus* species could synthesize many potent amphiphilic and surfactant lipopeptides comprising bacillomycins, iturins and mycosubtilin (GONG et al. 2015). In the same way, *B. subtilis* BJ-1 may produce approximately seven lipopeptide synthesis genes, encoding surfactin, fengycin, subtilin and bacilysin, which significantly inhibit mycelial growth of *P. oryzae* (HE et al. 2019). Soaking the seed with *B. subtilis* induced systemic resistance to the rice plants while promoting plant growth, and reduced the severity of disease by more than 70%, similar to the effect of fungicide tricyclazole (HE et al. 2019). A study by Rais et al. (2017) reported *Bacillus* spp. significantly colonized the rice plants and secreted multiple biocontrol determinants such as protease, glucanase, siderophores in the rhizosphere of different rice varieties. In another study performed by Suryadi et al. (2013) the use of *B. firmus* E65 and *B. cereus* II.14, and their combination conditions had a good effect in suppressing rice blast disease under greenhouse. Investigations from Sha et al. (2016) showed that the mixture of *B. subtilis* and *M. oryzae* improved the activity of peroxidase and polyphenol oxidase in rice plants, with significant activation of superoxide dismutase. In addition, the rice plants sprayed with *B. subtilis* alone inhibits the germination of *P. oryzae* spores, the length of the germ tube, the formation of appressoria, and degrades the structures of conidia and hyphae. Species of *Bacillus* are also capable of synthesizing some enzymes such as chitinase and b-1,3-glucanase having a strong lytic activity (SHAFI et al. 2017). These lytic enzymes have proved to be active in degrading fungal cell walls (LEELASUPHAKUL et al. 2006). The antagonistic *Bacillus* spp. could also elicit the antioxidant and defense enzymes such as SOD, POX, PPO, and PAL which are the

first line of defense against the plant pathogens (RAIS et al. 2017).

1.2.7.1. Morphology characteristic of *Bacillus* spp.

The Eubacteria from the Prokaryotes contain all the phytopathogenic bacteria, is divided according to the well-known Gram stain, into gram-positive (a single membrane) and gram-negative (2 membranes therefore no staining) (DESOIGNIES, 2010, p. 34). Gram + are divided according to whether their GC content is greater (Clostridium, Mollicutes, and Bacillus) or less (Actinobacteria) at 50% (DESOIGNIES, 2010, p. 34). The genus of *Bacillus* belongs to the Order of Bacillales and the family of Bacillaceae. The species are rod-shaped, endospore-forming aerobic or facultatively anaerobic, one of the largest, most ubiquitous, and currently comprises 268 species and 7 subspecies worldwide. Gram-positive organisms, *Bacillus* bacteria can produce more than two dozen antibiotics with an amazing variety of structures (STEIN, 2005, p. 845-857) (Fig.5 B). *Bacillus* species have long been used as biocontrol agents, mainly in the plant rhizosphere (THAKUR, 2016, p. 84). Only one endospore is formed per cell. The spores are resistant to heat, cold, radiation, desiccation, and disinfectants.

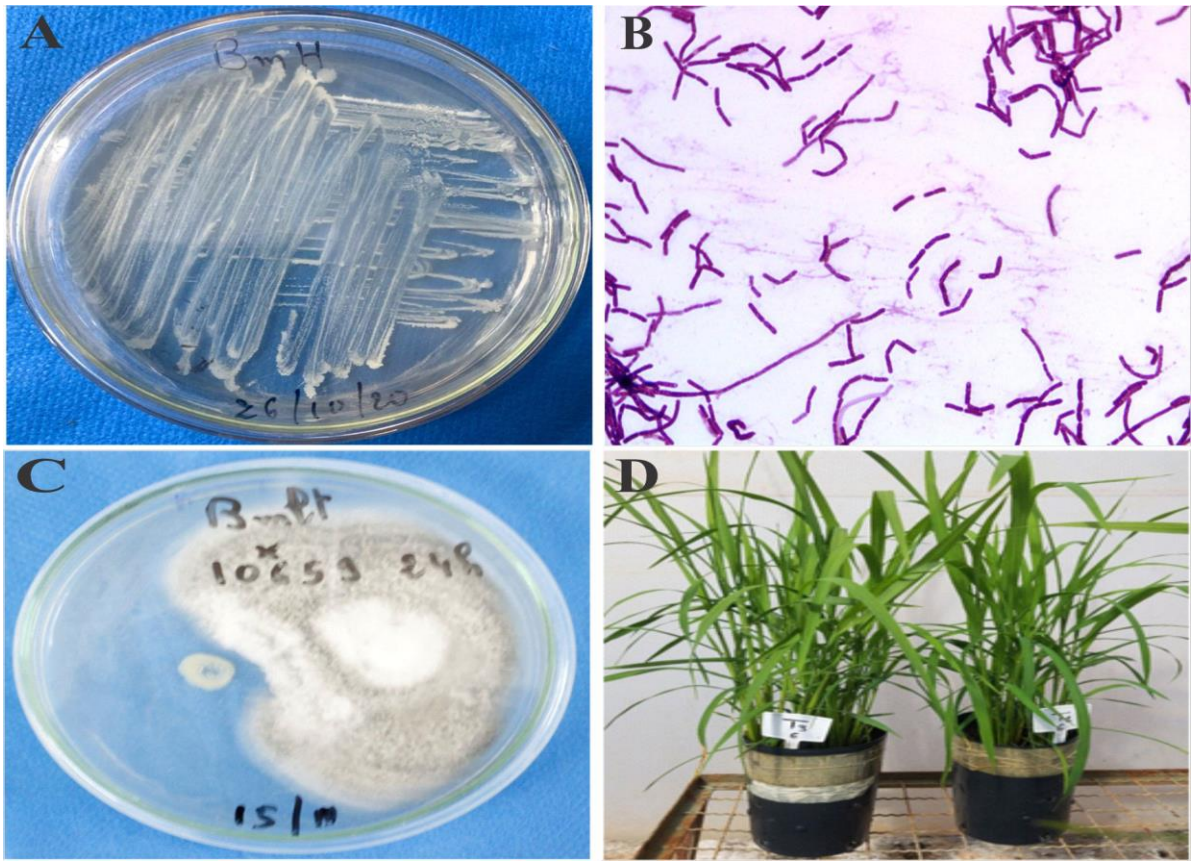

Figure 5. *Bacillus* sp. morphology, on planta, and *in vitro. Bacillus* sp. BMH growth on a Petri dish after 7 days of culture on Potato-Dextrose- Agar (PDA) medium at 25° C in photoperiod light and darkness (A). Endospores of *Bacillus* sp. on (B). Co-culture of *Bacillus* sp. BMH and *P. oryzae* to evaluate its antagonism system (C). Soil inoculated with *Bacillus* sp. BMH and negative control treatments were observed on the rice plants 45 days after sowing (D).

1.2.8. Antifungal properties of *Epicoccum* species

Epicoccum is ubiquitous fungi found in air, soil, decaying vegetation, and healthy tissues (BRAGA et al. 2018). *Epicoccum* is mainly known as a biocontrol agent against phytopathogens and for its ability to produce many secondary metabolites with the potential of biotechnological applications such as antioxidant, and antimicrobial compounds (PASCUAL, et al. 2000). According to Li et al. (2013), the production of the antifungal compound, the production of large spores with large amounts of stored nutrients, and its increased ability to survive in unfavorable climatic conditions make *Epicoccum* an attractive candidate as a bioagent. *Epicoccum*

sp. is a promising source for a wide variety of vital metabolites such as alkaloids, flavonoids, phenols, steroids and terpenoids (NISA et al. 2015). A recent study by Sena et al. (2013) indicated that *Epicoccum* spp. obtained from the phylloplane of rice plants, showed antagonistic potential against several pathogens such as *P. oryzae, R. solani, Sarocladium oryzae, Monographella albescens,* and *Cochliobulus miyabeanus*. It also could reduce rice leaf blast severity and induce plant defense responses by increasing the activity of peroxidase, β-1,3-glucanase, chitinase and PAL 24 hours after application (SENA et al. 2013). Koné (2016, unpublished data) found *in vitro* the ability of *Epicoccum* spp. strain BC706, to inhibit *P. oryzae* mycelial growth with 84.6% at 6 days incubation at 22° C. According to Hashem and Ali (2010), the use of *E. nigrum* involved the protection of cotton seedlings against *Pythium* damping-off and root-rot, but also enhanced their vigor and growth characteristics. In recent years, *E. nigrum* is more effective against conidia of *Monilinia* spp. applied the month before fruit harvest and gives the same effect as a fungicide (LARENA et al. 2009). Derbalah et al. (2011) evaluated that *E. nigrum* was effective against powdery mildew compared to other agents. A synthesis of the available study reported that *Epicoccum* spp. has proven to be a potent biocontrol agent against many phytopathogenic fungi and oomycetes, especially *B. cinerea*, *Claviceps africana* in sorghum, *Pythium* spp. on cotton, *R. solani* and *P. infestans* on potato plants, *S. sclerotiorum* in sunflower, phytoplasma in apple trees, and *Monilinia* spp. in peaches and nectarines (ELKHATEEB; DABA, 2019). A recent study by Braga et al. (2018) reported that *Epicoccum* spp. produced many secondary metabolites such as polyketides, polyketide hybrids, and diketopiperazines. In addition, many of these metabolites present some activities, such as antimicrobial, antioxidant, anticancer, and viral replication inhibition (BRAGA et al. 2018). Recently, *E. nigrum* has been used as a source of orsellinic acid and curvularin compounds, associated with silver nanoparticles (AgNPs) formation, which is shown to get potent antifungal activity against *Alternaria solani* (ABDEL-HAFEZ et al. 2017).

1.2.8.1. Morphological characteristics of *Epicoccum* spp.

Epicoccum belongs to the fungi kingdom, the Ascomycota Phylum, subphylum of Pezizomycotina, class of Dothideomycetes, order of Pleosporales, a family of Didymellaceae and *Epicoccum* genus (FATIMA et al. 2016; MCLAUGHLIN; SPATAFORA, 2015). Didymellaceae

family contains numerous plant pathogenic, saprobic and endophytic species associated with a wide range of hosts (CHEN et al. 2015). This ubiquitous mitosporic fungus is widespread in air, in soil, and on decaying vegetation, usually acting as a saprophyte or secondary invader of a plant's senescent tissues (BRAGA et al. 2018). The species of *Epicoccum* are found on different hosts such as plants, animals, marine organisms, fruiting bodies of other fungi (FATIMA et al. 2016). *Epicoccum* is characterized by dark sporodochia with branched conidiophores and mono- to polylactic, colorless conidiogenous cells that produce colored dictyoconidia (JAYASIRI et al. 2017) (Fig.6). The coelomycetous asexual stage is characterized by the production of pycnidia conidiomata with monophialidic, doliiform to flask-shaped conidiogenous cells that produce unicellular, hyaline conidia in culture or nature (CHEN et al. 2015). The genus of *Epicoccum* produces chlamydospores in culture (CHEN et al. 2015). Its colonies are often bright, red, orange, brown and yellow and can grow between -3 and 45°C with pH 3 - 4.5 (FATIMA et al. 2016). The conidiophores of *Epicoccum* are smaller in size (ranges from 15-25 microns), unremarkable and grouped in clusters. Spores are globose, dark brown, and muriform (septa in both directions, like a football) and there are often found as little black dots cultures media (FATIMA et al. 2016). Currently, *Epicoccum* genera count 18 species including *E. brasiliense, E. camelliae, E. dendrobii, E. draconis, E. duchesneae, E. henningsii, E. hordei, E. huancayense, E. italicum, E. latusicollum, E. layuense, E. mackenziei, E. nigrum, E. pimprinum, E. plurivorum, E. poae, E. sorghinum,* and *E. viticis* (BRAGA et al. 2018). Some species are endophytic commonly isolated from different sources in moderate frequencies (ARNOLD, 2007, p. 51-66). Some of these species have been described as plant pathogens like *E. sorghinum* on *Cucumis melo* (CHEN et al. 2015), and this last one is known as a highly variable species from endophytic to saprophytic lifestyles (BRAGA et al. 2018). *Epicoccum* spp. are also known for their ability to produce many pigments with hues in red-orange-brown-yellow spectra, mainly polyketides: flavipin (yellow), epicoccones A and B (brown), epicoccalone (yellow), epicocconone (fluorophore, weakly green fluorescent in water and strongly orange-red fluorescent in the presence of proteins), acetosellin (yellow), and orevactaene (orange); and carotenoids: b-carotene (yellow), c-carotene (orange), rhodoxanthin (red), and torularhodin (purple) (BELL; KARUSO, 2003; Braga et al. 2018). *Epicoccum nigrum* Link (syn. *E. purpurascens* Ehrenb. ex Schlecht) is a saprophytic ascomycete distributed worldwide

(FÁVARO et al. 2011). This species name should refer to only one lineage. However, morphologically and with genetic variation, *E. nigrum* presents two genotypes that may comprise more than one species (FÁVARO et al. 2011).

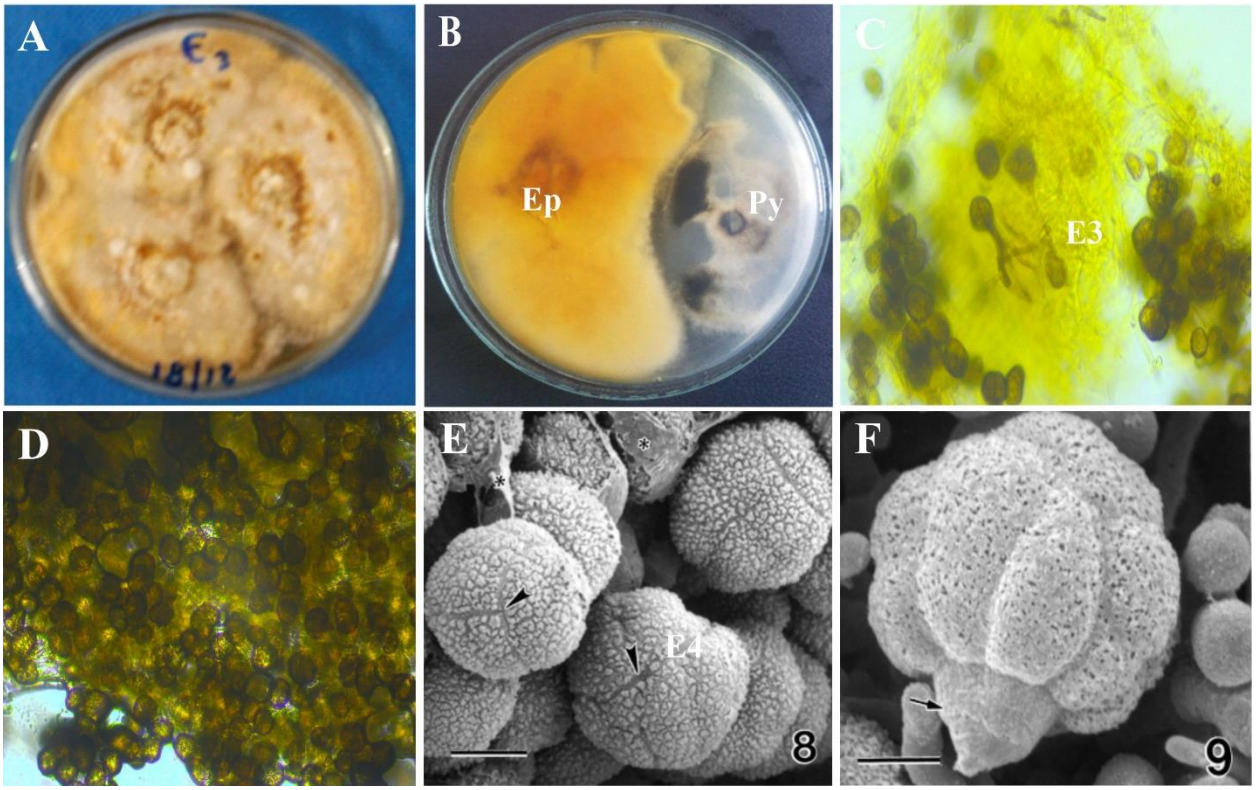

Figure 6. *Epicoccum nigrum* growth *in vitro* on PDA and OA medium. Conidia production on a petri dish after 15 days of culture on an oat-meal agar medium at 25°C in photoperiod light and darkness (A). *In vitro* co-culture of *E. nigrum* and *P. oryzae* on the potato- dextrose agar (PDA) medium (B). Conidium and mycelium of *E. nigrum* from *Vitis vinifera* (grapevine) plant and *Zea mays* were observed with optical microscopy in (C) and (D) respectively. (E and F): Scanning electron micrographs of *E. nigrum* conidium, note the smooth intersecting lines (arrowheads) its surfaces with the scale bars = 10 mm, and side view of a detached conidium respectively (MIMS; RICHARDSON, 2005).

1.2.9. Antifungal properties of *Penicillium* species.

Over the past 2 decades, endophytic *Penicillium* species have been investigated beyond their antibiotic potential and numerous applications have been reported. They have been investigated for agricultural purposes specifically against phytopathogens and insects as well as to reduce the pollution of agricultural farms by pesticides and heavy metals (TOGHUEO; BOYOM, 2020). *Penicillium* species are ubiquitous fungi that have been reported to colonize their ecological niches and protect their host plant against multiple stresses by exhibiting diverse biological functions and can grow over a wide range of conditions and environments (TSANG et al. 2018). The endophytic lifestyle has been reported to confer on *Penicillium* species the ability to protect its plants against biotic stresses and promote plant growth (WAQAS et al. 2015; LATIF et al. 2015). Endophytic fungi such as *P. citrinum* may play an important role in plant survival by enhancing nutrient uptake and producing growth-promoting metabolites such as gibberellins and auxins (KHAN et al. 2008). Indeed, *Penicillium* has become one of the genera of fungi best known for the production of bioactive compounds (LÚCIA; ENGUITA, 2016). For instance, *P. ruqueforti* induced high resistance in wheat plants grown on soil contaminated with heavy metals by restricting the transfer of heavy metals from soil to plants by secreting indole acetic acid (IAA) (IKRAM et al. 2018). The endophytic fungus *P. restrictum* has been reported to produce polyhydroxy anthraquinones compounds which are suggested to be the bacterial QS inhibitors (FIGUEROA et al. 2014). Nowadays, dozens of active compounds from endophytic *Penicillium* spp. have been reported (TOGHUEO; BOYOM, 2020). According to Altaf et al. (2017) species of *Penicillium* are known to enhance plant growth and yield by providing available phosphorus and other plant-growth hormones such as IAA (indole 3- acetic acid) and gibberellins. Some of these species can synthesise nanoparticles that have attracted considerable interest in nanotechnology (TOGHUEO; BOYOM 2020). Microbial endophytes like *P. chrysogenum* and *P. crustosum* have played a vital role in enhancing *Teucrium polium* plant growth through their varying phosphate solubilization, enzymatic and antimicrobial activities (HASSAN, 2017, p. 687-695).

1.2.9.1.Morphological characteristics of *Penicillium* spp.

Penicillium belongs to the class Plectomycetes that originally contained all ascomycetes which produce their asci within a cleistothecium, i.e. “a closed case”(WEBSTER; WEBER, 2007). It belongs to the Eurotiales Order in which the conidia commonly produced from phialides is a relatively large order of Ascomycetes with members frequently having poa positive and negative impacts on human activities (Fig7). Eurotiales is one of the most important groups of fungi because it contains many ubiquitous and easily recognizable species, like the anamorphic of *Penicillium* (WEBSTER; WEBER, 2007). It belongs to the family of Aspergillaceae, and contain a large number of species possessing a worldwide distribution and a wide range of ecological habitats (TSANG et al. 2018). Its species are ubiquitous and can be found in the air, soil, vegetation, and indoor environments. Species of *Penicillium* live in soil and whatever organic matter; on seeds and grains thermotolerant, xerophiles (low availability of water), produce a vast array of secondary metabolites, enzymes, mycotoxins (TSANG et al. 2018). *Penicillium* its sexual morphs *Talaromyces* and *Eupenicillium* are one of the largest groups of fungi, composed in the most recent list from 2014, 354 *Penicillium* species were recognized and the current list includes 483 species and 88 *Talaromyces* species, found in different environments (HOUBRAKEN et al. 2020; TOGHUEO; BOYOM, 2020). The teleomorph genera historically associated with *Penicillium* are *Talaromyces* and *Eupenicillium* (HOUBRAKEN et al. 2020; SAMSON et al. 2011). According to Webster and Weber (2007), the teleomorph of *Penicillium* spp. has been found only for about 31%. These species have been isolated as endophytes of multiple and various plant species (TOGHUEO; BOYOM, 2020). It is a decomposer of organic substances, while some species are pathogenic in the form of rots of food crops, especially in post-harvest by secreting different varieties of mycotoxins (ALTAF et al. 2017). It has a huge economic impact on human life (TSANG et al. 2018). For instance, the significance of this genus became famous with the discovery of the antibiotic penicillin, which transformed medical approaches to treating bacterial pathogenic *Staphylococcus aureus* diseases that were a major scientific and medical breakthrough in treating microbial infections (FLEMING, 1929, p. 245-250). Also, these species are vital to the food industry and quite a number of them are exploited to produce fermented food such as cheeses (e.g. *P. roqueforti*), sausages (e.g. *P. nalgiovense*) (TSANG et al. 2018). Its species applications are

diverse, including the production of enzymes or primary and secondary metabolites, and direct colonization and modification of foodstuff (WEBSTER; WEBER 2007). Two *Penicillium* spp. (*P. roqueforti* and *P. camemberti*) are important in specialized cheeses for blue-veined cheeses, and white mold cheeses. Species of *Penicillium* are not as thermotolerant, with only relatively few species capable of growing at 37°C (WEBSTER; WEBER, 2007).

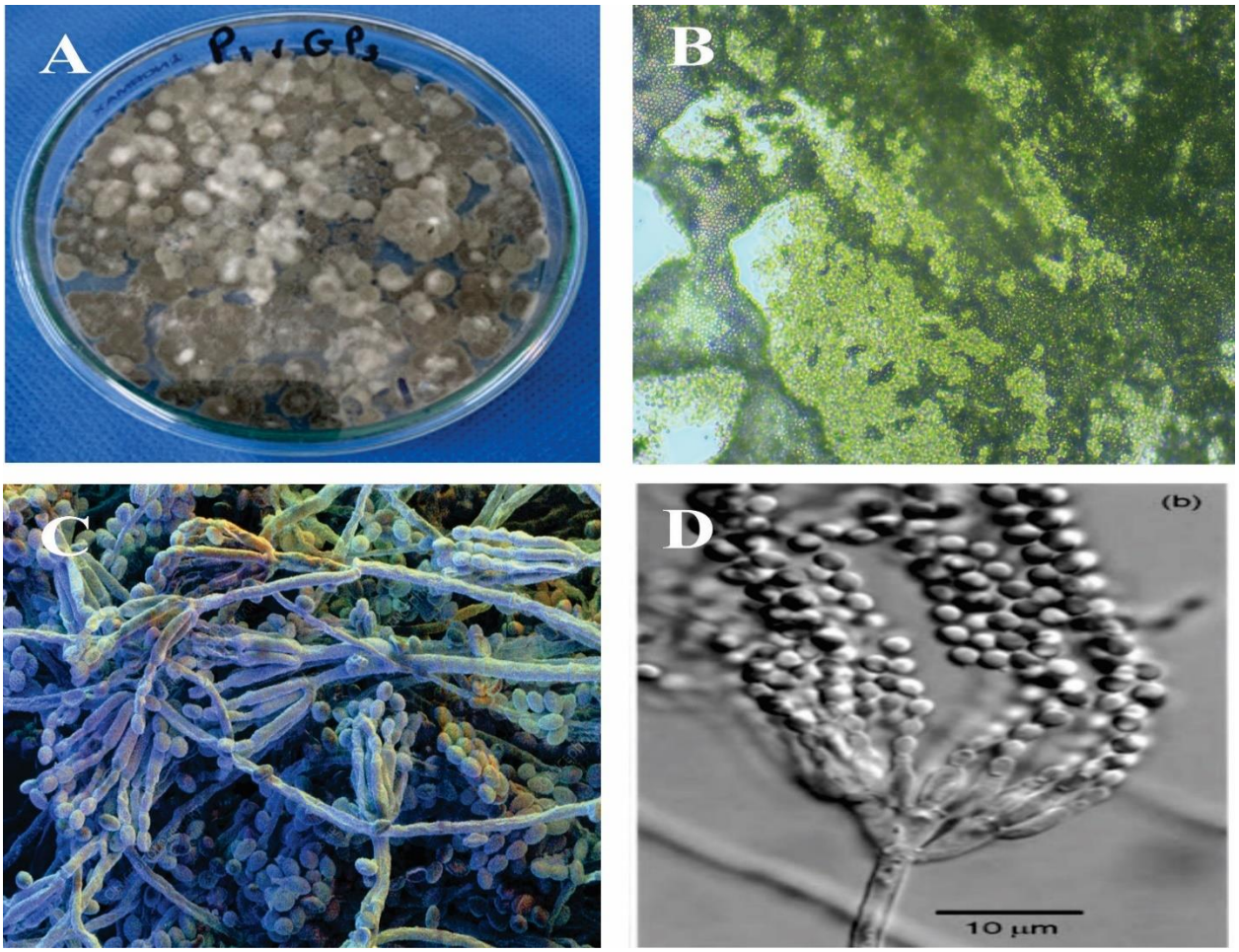

Figure 7. *Penicillium* spp. morphology. Conidia production on a Petri dish after 15 days on potato-dextrose-agar (PDA) medium at 25° C in photoperiod light and darkness (A). Conidium of *Penicillium* sp. from *Vitis vinifera* (grapevine) plant observed with optical microscopy in (B). Colored Scanning Electron Micrograph (SEM) of the mycelium and fruiting bodies of the fungus *Penicillium* sp. from science photo. com/media/13549/view (C). *P. chrysogenum*, the conidiophore tip that gives rise to phialides (D).

1.2.10. Endophytic biological control agents

The term endophyte is derived from two Greek words: ‘endo’ = ‘endon’ meaning within, and ‘phyte’ = ‘phyton’ meaning plant. This term applies to the organisms living within the plants (LATA et al. 2018). Endophytes are fungi or bacteria that at a certain stage or all stages of the life cycle colonize in healthy plant tissues without causing obvious damage (KUSARI et al. 2012; ZHOU et al. 2018). In another word, an endophytic fungus is a fungal microorganism, which spends the whole or part of its life cycle colonizing inter and/or intracellular within healthy tissue of host plants, usually causing no noticeable symptoms of disease (PETRINI 1991, p. 245-250, TOBERGTE; CURTIS, 2013). These microorganisms which asymptomatically inhabit plant tissues have been isolated from many species of woody plants and grasses (FÁVARO et al. 2011) and their diversity is greater in tropical regions (ARNOLD, 2007, p. 51-66). Endophytic fungi are widely reported for their ability to aid in the defense of their host plants, the production of valuable bioactive molecules, as well as their biofertilizer ability (YAN et al. 2019). Currently, endophytes have been isolated from both aboveground and belowground tissues of many plant species, with a characteristic of high species diversity consisting of more than 30 fungal species per plant species (NISA et al. 2015; YAN et al. 2019). Their enormous biological diversity, associated with their ability to synthesize several bioactive secondary metabolites has prompted research on these endophytes (KUSARI et al. 2012). Humans also maintain a symbiotic association with their gut microbial flora, which is crucial for nutrient uptake and the development of the innate defense system (HUGHES; SPERANDIO, 2008).

The advantage that endophytes have over other biocontrol agents is their ability to colonize plant's tissues (SHAH et al. 2021). The host tissue colonization by beneficial endophytes may be local or systemic, inter- or intracellular, and the effects on the host also vary from asymptomatic to mutualistic symbioses (BOYLE et al. 2001). This symbiosis allowed the host plant protects and nourish the fungus which in return produces bioactive substances, such as plant growth regulators, secondary metabolites to foster the growth and competitiveness of the host in nature and contribute positively to their host plant (SCHULZ; BOYLE, 2005). As mentioned earlier, microbial endophytes present themselves as a producer of stimulators of many bioactive metabolites such as

flavonoids, peptides, alkaloids, steroids, phenolics, terpenoids, flagella, QS, siderophore auxin, cytokinin and gibberellins, antioxidants (AFZAL et al. 2019). Also, QS systems have been shown to control biofilm formation and swarming motility (HUBER et al. 2001).

1.2.11. Mechanisms of plant growth promotion microbes

Plant growth promotion may involve nutrient uptake, production of growth regulators like auxins, cytokinins, and gibberellins in the direct mechanisms (ALORI et al. 2017; VAN DER LELIE et al. 2009). The symbiosis of biofertilizers producing endophytic fungi with crops can be a promising strategy to overcome the adverse effects of abiotic stresses (KHAN et al. 2015). This association could ameliorate plant growth during harsh environmental conditions (Khan et al. 2015). The most commonly microbial endophyte used as biofertilizers, biocontrol include *Bacillus* spp., *Pseudomonas* spp., *Streptomyces* spp., *Trichoderma* spp., and *Mycorrhizas* (ALORI et al. 2017). Some PGPB could directly promote plant growth and are classified into biofertilizers, rhizoremediators, phytostimulators, or stress controllers, some of them act indirectly through the control of plant diseases, and others use both mechanisms (GAMALERO; GLICK, 2011; VELIVELLI et al. 2014). According to Saranraj (2014), *Bacillus* is the most abundant genus in the rhizosphere that trigger plant growth, resulting in the synthesis of plants metabolites such as auxin, cytokinin and gibberellins. In addition, Wang et al. (2018) suggested that *B. subtilis* promote plant growth and increase nitrogen (N) uptake, phosphate solubilization, siderophore and phytohormone production. A study conducted by Zhang et al. (2009) indicates that *B. subtilis* can permit to increase in the accumulation of Fe in plant tissues while improving the photosynthetic capacity of the plant. It has been shown that the application of fertilizers may not solve the nutritional problems of plants, as phosphorus (P) can easily bind to cations and become insoluble after its use in the soil (ADESEMOYE; KLOEPPER, 2009). Several species of bacteria are involved in phosphate solubilization including *Pseudomonas, Bacillus, Rhizobium, Enterobacter,* and *Streptomyces* (VELIVELLI et al. 2014), *Azospirillum, Azotobacter, Burkholderia* and *Serratia* (MEHNAZ; LAZAROVITS, 2006). Further research showed that volatile organic compounds (VOCs) such as 2,3-butanediol and acetoin had been released by some endophytes bacteria which trigger the great

level of plant growth promotion (RYU et al. 2003). It has been shown that endophytic *Bacillus* sp., *Pseudomonas* sp., *Flavobacterium* sp. would promote plant growth through N fixation mechanisms, solubilization and transformation of minerals, siderophores production, use of 1-aminocyclopropane-1-carboxylate (ACC) acid as the sole source of nitrogen (RAJKUMAR et al. 2009). Also, Xie and Yokota (2005) reported the ability of some endophytic bacteria from rice to be N-fixer such as *Beijerinckia, Methylocystis, Methylosinus, Methylobacterium, Bradyrhizobium,* and *Rhodopseudomonas*. On the other hand, fungi also could facilitate the essential biochemicals transport and assimilate N and P for plant growth and may help to counteract stressful events (KHAN et al. 2015). Further research has demonstrated that *Colletotrichum tofieldia* in roots could spread systemically into shoots, transfer phosphorus to shoots, promotes plant growth, and increase fertility only under low phosphorus conditions. Sessitsch et al. (2012) reported that the endophytic bacteria community that colonizes rice roots have a high potential for plant-growth promotion, improving plant stress resistance, control of pathogens, and bioremediation. Their prominent features included flagella, plant-polymer-degrading enzymes, protein secretion systems, iron acquisition and storage, QS, and detoxification of ROS.

1.2.12. Electron microscope for samples examination

In order to study microorganisms (antagonists, pathogens) interactions with plants or between pathogens and antagonists, histological and ultrastructural studies are important to provide accurate information about the development of their interactions during the infection or plant growth process. So, in histological and molecular studies of the interaction between rice (*Oryza sativa* L.), rice blast fungi, and endophytic organisms such as *Bacillus* sp., *E. nigrum*, and *P. citrinum*, using Scanning microscopy, Epifluorescence microscopy, and Confocal microscopy for more understanding of the interaction between the structures of the pathogen, the BCA, and host cells.

1.2.12.1. Scanning Electron Microscope (SEM)

The SEM is a helpful tool to study microorganisms (fungi, oomycete, and bacteria), their interaction with other organisms or with plants (ALVES et al. 2013). It allows studying of several aspects of the morphology, such as surface details, fungi parasitism, and saprophytism (ALVES et al. 2013). Nowadays, SEM is utilized not only in materials, chemical and physic sciences but also in diverse fields such as medical sciences and biology (KASHI1 et al. 2014). The high spatial resolution of an SEM makes it one of the most versatile and powerful equipments available for the examination and analysis of a wide range of the microstructural characteristics of specimens at the nanometer to micrometer length scale (KASHI1 et al. 2014). It gives the three-dimensional aspect to the images, large magnitude of increase from 10 to 1000000 times, rapid processes of image digitalization (ALVES et al. 2013). The images generated by SEM are used to broaden the understanding of the interactions between organisms, and between these organisms and plants. It is also used for better details for fungi and oomycetes identification (ALVES; POZZA, 2009). One of the big challenges of SEM in interpreting images of biological samples is being able to distinguish features that reflect the natural structure from those created artificially during processing (KASHI1 et al. 2014). To use SEM, every sample must be completely dry and free of any organic contaminants (KASHI1 et al. 2014).

1.2.12.2. Epi-Fluorescent Microscope (EFM)

In order to study plant-pathogen interactions, histological studies are important to provide accurate information about the development of the fungus during the infection process (ZHANG et al. 2001). Several methods have been developed to analyze the viability of individual cells as well as to detect specific populations. Most of these methods are based on the detection of fluorescence. Epifluorescence microscopy (EFM) is commonly used for the detection of these events.

1.2.12.3. Confocal Laser Microscope (CLM)

CLM is a major advance upon normal light microscopy, allowing one to visualize not only deep into cells and tissues, but also to create three-dimensional images and to follow specific cellular reactions over extended periods (HIBBS, 2004, p. 467). The main advantage of a confocal microscope is its ability to optically section thick specimens (MASTERS; SO, 2001). There are two enhancements of the imaging characteristics of a confocal microscope as compared with light microscopes, first, it enhanced lateral resolution, and secondly enhanced axial resolution (MASTERS; SO, 2001). The confocal approach provides a slight increase in both lateral and axial resolution (Paddock, 2000). The confocal approach has facilitated the imaging of living specimens, enabled the automated collection of three-dimensional data in the form of Z-series and improved the images of multiply labelled specimens (PADDOCK, 2000,p. 127-149). The development of confocal microscopes was driven largely by a desire to image biological events as they occur *in vivo*. In CM, the image is either built up from the output of a photo-multiplier tube or captured using a digital charge-coupled device (CCD) camera (PADDOCK, 2000, p. 127-149).

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PART II- EXPERIMENTAL STUDIES

CHAPTER I- Microscopic and molecular studies in the biological control of rice blast caused by *Pyricularia oryzae* with *Bacillus amyloliquefaciens* BMH under greenhouse conditions

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ABSTRACT

Rice feeds around half of humanity mainly in Asia, South America, and Africa. *Pyricularia oryzae* Cav. (syn. *Magnaporthe oryzae* Couch) is the causal agent of blast rice, the most important rice disease worldwide. In this study, we evaluate the efficacity of the *Bacillus amyloliquefaciens* BMH strain in the control of *P. oryzae,* as well as in the growth promotion, and productivity of the cultivar BRS.MG Caçula. Also, we evaluate the ability of *Bacillus* BMH to colonize tissues using microscopy, and genes expression under rice plants developed in greenhouse conditions. The soil and seedlings were inoculated with *Bacillus* BMH and the seedlings were sprayed with *P.* oryzae isolate A10965. The severity evaluation and microscopic analysis showed that BMH was able to reduce rice blast disease severity in 53.1%, 50%, and 37.5% for soil inoculation, preventive control, and the mixture of pathogen-BMH treatments, respectively, 28 DAI. Height rice plants were higher

when *Bacillus* BMH was inoculated in soil with 28.5 cm compared to the control with 25.7 cm 63 days after sowing (DAS). Likewise, the number of tillers was higher with 12.6 for BMH compared to 9.1 for control. The weight of grains was 10.2 g for BMH treatments against 7.1 g for the control treatment. The Confocal Laser Microscopy (CLM) and Scanning Electron Microscopy (SEM) showed the colonization of rice root vascular tissues as well as foliage by *Bacillus* BMH cells, which indicates its endophytic potential. Soil inoculation with BMH or sprayed on the surface of leaves resulted in the stimulation of rice defense mechanisms against blast disease by upregulating the rice β-1,3-glucanases (OsGLN1) and rice acidic pathogenesis-related (OsPR1a) genes involved in plant normal growth, development, and play a key role in defense against fungi. The results obtained in this study demonstrate the beneficial interaction between rice plants and *B. amyloliquefaciens* BMH.

Keywords: Rice blast, *Bacillus amyloliquefaciens*, rice blast severity, induced resistance, growth promotion.

1. INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food for more than half of humanity, mainly in Asia, Latin America, and Africa (Fageria et al. 2014). Rice occupies a preponderant place in the food diet of Africans and it represents more than 25% of the total cereals consumed following the maize (MENDEZ DEL VILLAR; BAUER, 2013). However phytosanitary conditions are factors that determine the production of rice worldwide, among them diseases determine both quality and productivity losses (WILSON; TALBOT, 2009).

Pyricularia species are cosmopolitan and multi-host pathogens that infect more than 50 different species of cereals and grasses (GLADIEUX et al., 2018) including economically important cereal crops, such as wheat, barley, and millet (FERNANDEZ; ORTH, 2018). *Pyricularia oryzae* Cavara (syn. *Magnaporthe oryzae* B. Couch:) (WEBSTER; WEBER, 2012) is a hemibiotrophic seed-borne and airborne plant pathogen that is among the most destroyer

pathogen of rice (ZHANG et al. 2018). Considered as one of the most production constraints particularly devastating in lowland and upland rice crops (GNANAMANICKAM; MEW, 1992), it can infect all aerial rice tissues, including leaves, stem, neck, panicles, and recently the root (AGRIOS, 2005, p. 922; ASIBI et al. 2019; RIBOT et al. 2008). This pathogen has developed different strategies to overcome the barriers it encounters during rice infection (GLADIEUX et al. 2018). The severity of rice blast has increased due to intensive rice cultivation which favors its development (ALI; NADARAJAH, 2014). The severity is generally increased by the excess of nitrogen fertilization (ASIBI et al. 2019), prolonged leaf wetness, not only but also by rainfall (AGRIOS, 2005, p. 922), and the increasing canopy density (KÜRSCHNER et al. 1992).

Management of rice blast has been carried out with fungicides treatment because resistant cultivars become vulnerable within a few years after release (BALLINI et al. 2008; CAWOY et al. 2009). However, the use of chemical fungicides leads to environmental threats (risks of toxicity) and the appearance of resistant strains of the pathogen (CHUMA et al. 2011; NGUYEN et al. 2016; RAVELOSON et al., 2013; WILSON; TALBOT 2009). Aware of these potential risks, there is a need to shift to agricultural production practices that are more sustainable, advantageous, respectful of the environment, and allow crops to produce well (DUBEY et al. 2015; MORICCA et al., 2015). Biocontrol agents are becoming a promising means to control fungal diseases and reduce dependence on chemical fungicides, and also for promoting plant growth (BOČEK et al., 2012; PII et al. 2015; WANG et al. 2018).

Bacillus genus is a gram-positive bacterium, that has a unique ability to grow fast, is resistant to adverse conditions as well as has a broad spectrum of biocontrol agent ability (SHAFI et al. 2017). It has been found as soil-inhabiting or as epiphytes and endophytes in the spermosphere and rhizosphere (BERIĆ et al. 2012). Several species of *Bacillus* produce numerous secondary metabolites and have been found to show antifungal activity against different plant pathogens (ARGUELLES-ARIAS et al. 2009). According to Saranraj (2014), *Bacillus* is the most abundant genus in the rhizosphere that shows plant growth promotion activity, resulting in the synthesis of some metabolites such as auxin, cytokinin, and gibberellins. Nowadays, plant growth-promoting rhizobacteria (PGPR) are being widely used as an alternative to chemical fungicides and fertilizers (TOBERGTE; CURTIS, 2013) due to their ability to enhance plant growth by various mechanisms.

Also, the volatile compounds produced by *B. subtilis* may play an important role in plant growth promotion, and activation of plant defense mechanisms by triggering induced systemic resistance (ISR) in plants (COMPANT et al. 2005; SHAFI et al. 2017). Some investigations reported that *Bacillus* species synthesize many potent amphiphilic and surfactant lipopeptides comprising bacillomycins, iturins, and mycosubtilin (GONG et al. 2015).

Plants recognize beneficial and pathogenic microbes through molecular patterns associated with microbes like a microbe or pathogen-associated molecular patterns (MAMPs or PAMPs), which include microbial secondary metabolites (BUCHANAN et al. 2015). Upon colonization of tissues by specific beneficial microbes or infection with pathogens, plants can develop induced resistance (CHAGAS et al. 2018). The first branch uses transmembrane pattern-recognition receptors (PRRs) that recognize conserved PAMPs, leading to an immune response called PAMP-triggered immunity (PTI) or even MAMP-triggered immunity (MTI) (BUCHANAN et al. 2015; CHAGAS et al. 2018). During ISR, plants can defend themselves against pathogens through the production of secondary metabolites. Plant ALD1 is responsible for triggering basal defense response and systemic resistance against pathogen infection and is involved in the production of pipecolic acid and other compounds for systemic resistance (JUNG et al. 2016). In rice plants, 14 genes encoding putative β-1,3-glucanases that are involved in plant defense and development have been sequenced (WAN et al. 2011). Also, Pathogenesis Related (PR) proteins are defined as plant proteins that are induced in pathological or related situations, and the defense mechanisms have been identified in many plant species (WU et al. 2011). Some of them function as chitinases and glucanases degrading fungal cell wall structural polysaccharides and impairing fungal growth (BUCHANAN et al. 2015). The WRKY gene family has been suggested to play an important role in the regulation of transcriptional reprogramming associated with stress responses in plants (CHEN et al. 2012). In rice, the OsWRKY28 transcription is upregulated after infection with the rice blast fungus *P. oryzae* (CHUJO et al. 2013). The modification of the expression and/or the change of activity of the WRKY genes would contribute to the development of various signaling pathways and regulatory networks (CHUJO et al. 2013).

To study plant-pathogen and bioagent interactions, microscopic studies are important to provide accurate information about the development and tissues colonization of the biocontrol

agent, and pathogen during the infection process. Thus, the objectives of the current study were: (i) evaluate the efficiency of the selected *Bacillus* strain BMH on rice blast disease suppression, (ii) evaluate its ability to stimulate rice plant defense mechanisms against blast disease (iii) determine its interaction with rice plant (vi) evaluate the growth promotion and grain production of rice cultivar BRS.MG Caçula under greenhouse conditions. In the present study, we tried to develop management strategies aimed at reducing the use of chemical fungicides and fertilizers, and therefore their environmental impact using the bacterial biocontrol agent *Bacillus* sp. BMH against rice blast disease.

2. MATERIAL AND METHODS

2.1. Plant material and microorganisms

A rice cultivar BRS.MG Caçula, which is considered very susceptible to *P. oryzae* in climatic conditions in Minas Gerais state, Brazil, was used in this study. *Pyricularia oryzae* strain A10659 obtained from the Laboratory of Electron Microscopy and Ultrastructure Analysis, Department of Phytopathology (DFP), Lavras, Brazil collection was used in this study. *Bacillus* sp. strain BMH obtained from the Molecular Phytopathology Laboratory of the Department of Phytopathology (DFP) was used in the experiments. By 16s rRNA sequencing, BMH isolate was grouped as related species into the operational group *Bacillus amyloliquefaciens* with KU207996 as accession number is. The identity between BMH with *B. amyloliquefaciens* DSM 7 (type strain) was 99.69%.

2.2. Microorganisms mass production, plant growth conditions, and inoculation

The assay was conducted to evaluate the effectiveness of *Bacillus amyloliquefaciens* BMH against foliar rice blast. Rice seedlings were prepared by sowing the seeds of BRSMG Caçula variety in a pot filled with 800 g of the mixture of sterile sand and substrate soil and kept in a greenhouse controlled at 30 °C maximum temperature. This substrate Trosptrato® HA Hortaliças was composed of Pinus bark, vermiculite, 14.16.18 fertilizer, potassium nitrate, simple

superphosphate and peat. *Bacillus amyloliquefaciens* BMH was grown in Nutrient Agar (NA) medium for two days in the rotary shaker at 140 rpm at 28 °C and the concentrations adjusted to optical densities (OD_{600nm}) = 0.6 -1 diluted in water until final concentration. Thirty-five ml of the *B. amyloliquefaciens* BMH was drenched manually in the soil of each pot of the treatment at the moment (T3) and x hours before (T6) rice sowing (Table 1). For foliar control treatments, 20 ml of the *B. amyloliquefaciens* BMH and pathogen were sprayed for treatment at 18 days after sowing (DAS) on rice plants at the stage of one shoot with three leaves (seedling growth stage). Plants were irrigated to keep the lowland conditions and optimum moisture level as the requirement of the rice plants (FAGERIA et al. 2014). *Pyricularia oryzae* was grown in PDA and oat-agar (OA) medium for conidia mass production at 25 ± 2 °C for 15 days. The inoculum solution was prepared using sterilized water containing x ml of Tween 20® and adjusted for the concentration of 1×10^5 conidia mL^{-1}.

The fertilizer NPK 20-5-20 was applied at the recommended rate (90 kg. ha-1), with recommended nitrogen rate (40 kg. ha-1) applied twice (20kg at 15 days after sowing and 20vkg at the stage of tillering in 45 days)., rice seedlings were incubated in a growth chamber between 80 to 100% relative humidity at 25°C in the darkness for 24 hours to stimulate the infection after pathogen and BMH inoculation on leaves surfaces before transferred in the greenhouse. The pots with a surface of 103.82 cm^2 and a volume of 1765.77 cm3 were used for the rice seedlings.

The experiment was designed in complete randomized design (CRD) in six treatments and seven replications, each pot containing three plants. Two (2) replicates (pots) were used for microscopy studies, and five replicates to evaluate the disease severity and plant growth promotion.

Table 1- Schedule of *Bacillus amyloliquefaciens* BMH application in rice plants to control rice blast caused by *Pyricularia oryzae*.

Treatments/ Designation	Inoculation time	Plant growth stage
T1- Negative control (no pathogen)	Water 18 DAS	Beginning of tillering
T2- Positive control (only the pathogen)	*P. oryzae* 18 DAS	Beginning of tillering
T3-Bacillus positive treatment	*Bacillus* at sowing	Soil before sowing
T4- BMH positive (soil inoculation)	A mixture of *Bacillus* x *P. oryzae* 18 DAS	Beginning of tillering
T5- BMH and pathogen later	*Bacillus* first, and *P. oryzae* 24 hours after 18 DAS	Beginning of tillering
T6- BMH in soil and pathogen later	Soil inoculated with *Bacillus* before sowing and *P. oryzae*	Soil before sowing

DAS = day after sowing.

2.3. Assessment of blast disease, growth attributes and yield

Despite the advances in software and imaging-based tools to automatically assess the disease, qualitative or quantitative visual estimation remains the most common method used for severity assessment under controlled conditions as well as the natural environment (PONTE et al. 2017). Leaf blast disease severity was evaluated on three leaves of the three plants and was recorded at 7 days after inoculation (DAI) and every 7 days until leaves senescence using visual quantification based on a diagrammatic scale of 0 – 9 developed by the International Rice Research Institute, Philippines (REDOÑA, 2013, p. 52) (Fig 8). The reduction in leaf blast severity was calculated using this formula (LBS %) = (100 − (severity of the treatment × 100 / severity of the control)). The disease score data were converted to the area under the disease progress curve (AUDPC) according to the formula described by Madden et al. (2007).

The heights (cm) of the plants were measured and the number of tillers per plant was counted from 18 days after sowing and every 15 days until the number of tillers was stable. For the production data, the number of panicles per plant and the weight of grain were also measured.

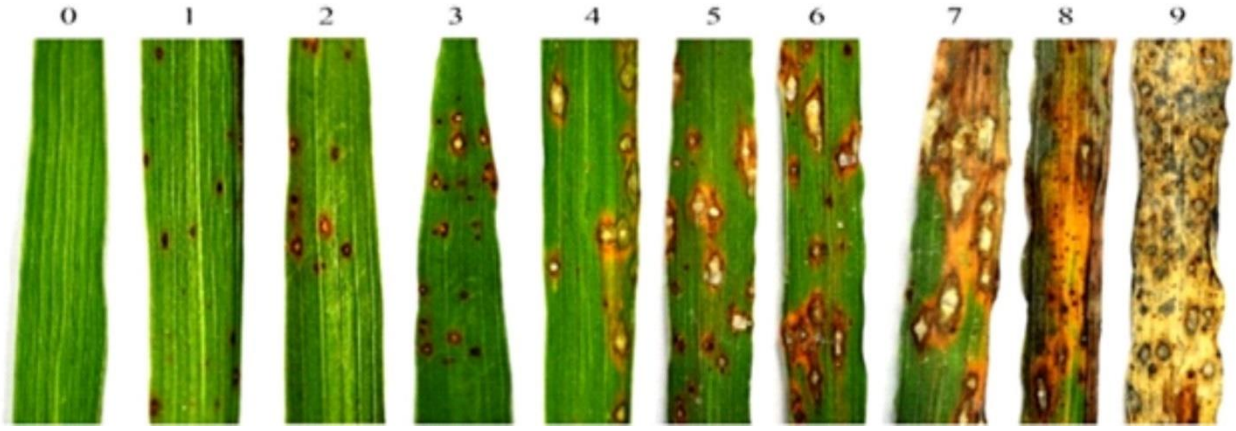

Figure 1- Schematic diagram of mechanism with the index value for scoring rice blast disease on foliage.

2.4. RNA extraction and real-time quantitative PCR

The seedlings of 48 HPI for *P. oryzae* and *B. amyloliquefaciens* BMH foliar treatment and 20-day old seedling for *Bacillus* soil inoculation were collected. The leaves of rice seedlings after inoculating with *Bacillus* and *P. oryzae* were collected at the sunny light at 11 am. Rice leaves of 4 replicates of each 5 treatments were ground in a mortar and pestle with liquid nitrogen and the powder was used for RNA extraction using TRIzol® reagent (Invitrogen). Total RNA concentration, quality, and yield of the extracted RNA were assessed through gel electrophoresis and UV spectrometry (Nanodrop 1000, Thermo Scientific). Then RNA was treated with RNase free DNase, and the cDNA synthesis was done using QuantiTect® Reverse Transcriptase kit. More details on this protocol are provided in Appendix C. Five genes identified in the rice genome responsible for conferring resistance to the blast disease were studied according to the literature consulted (Table 2). A mixture of 2.5 µL SYBR-GoTaq® qPCR Master mix, 0.4 µL of primers, 1.0 µL of cDNA, and 0.7 µL of water was used to determine the expression of rice resistance genes. The amplification conditions were two stages of 50 °C for 2 min and 95 °C for 10 min (initial activation), 40 cycles of 15 seconds at 95 °C (denaturation) and 1 min at 60 °C (annealing/extension), 15 seconds at 95 °C, 1 min at 60 ºC and 1 second at 95 ºC (melting curve). The accumulation of transcripts for each gene was normalized using the expression of the

constitutive gene 7Ubiq which is constitutively expressed in rice plants using QuantStudio™ design and analysis software. Relative quantification of gene transcripts was carried out using the method $2^{-\Delta\Delta Ct}$ (LIVAK; SCHMITTGEN, 2001).

Table 2- Primers used in molecular biology study.

Locus	Primer name	Primer sequences
ALD1 gene	OsALD1	Forward: 5'-CCCACATTTCCGGGGTACGTGG-3' Reverse: 5'-CGGAAATGTGGGATCTTGGA-3'
Acidic pathogenesis-related (PR) protein	OsPRI1a	Forward: 5'- GTCTTCATCACCTGCAACTACTC -3' Reverse: 5'- CATGCATAAACACGTAGCATAGC-3'
Basic pathogenesis-related (PR) protein	OsPRI1b	Forward: 5'-ATGGTAGCCGCCATGGCACTCC-3' Reverse: 5'- GCCGCTTCTCTGGCTGGCGTA-3'
Endo-1,3-β-glucanase gene	OsGLN	Forward: 5'-GACCATGTCAATGCGAATAAGG-3' Reverse: 5'- GCATTTTCAAAGCACAATGTGCC-3'
WRKY gene	OsWRKY28	Forward: 5'-AAGAACCTGGCCGAGCAGA-3' Reverse: 5'-TCAGTTCTTGGTCGGCGAGA-3'
Ubiquitin gene	7Ubiq	Forward: 5'-AGCACCTTCCAACAGATGTGGATCT-3' Reverse: 5'- CAAATACTTGACGAACAGAGGC -3'

2.5. Confocal Laser Microscopy (CLM) sample preparation

The rice leaves inoculated with microorganisms (pathogen or BMH) were cut in 4 × 4 mm pieces were prepared according to Moreira et al. (2019). The leaves fragments and roots of 3 plants (9 leaves) were used for the analyses. The tissues were placed within ELISA plates well containing KOH 10%, followed incubation at 10 °C for 4 days in darkness. The sample was then washed with phosphate buffer solution (PBS) and replaced with a new KOH 10% solution for 4 days again. It was then washed with PBS and fragments were transferred to ELISA wells with clarifying mix [Urea 6M, Glycerol 30% (v/v) and Tween 20 0.05% (v/v)]. After 4 days in clarifying mix at 10 °C and darkness, washed with PBS, and incubated in a new clarifying mix for 4 days. It had been washed again with PBS, and 100 µL Alexa488–WGA 10 µg mL-1 were added to observe the root or leaf surfaces and SYTO 9 to observe *Bacillus* cells. According to McGoverin et al. (2020), SYTO 9 binds to nucleic acid in the green fluorescent dye to see *Bacillus* cells. ELISA plates were

wrapped with aluminum foil and kept in a vacuum for 1 h. In these assays, the image was obtained using the Confocal Microscope, model LSM 780, and Zen software (Carl Zeiss).

2.6. Scanning Electron microscopy sample preparation

The collection of rice plant leaves, roots was done 21 DAS or 3 days after pathogen inoculation (Table 2). The tissues of interest were selected and cut into pieces of a maximum of 2 cm x 2 cm (to facilitate the stub mounting) before putting them in Karnovsky fixative for 24 hours. The samples were washed with cacodylate buffer and transferred to sequential dehydration in 25%, 50%, 70%, 90% acetone for 10 min each, and 100% 3 times for 10 min each. Then, the samples were transferred to the critical point dryer to complete the drying process with carbon dioxide as the transition fluid (Alves et al. 2013) and mounted on aluminum wheels with a double stick carbon tape glued to a film of aluminum foil while taking care to keep the area to be observed upwards. Thereafter, the specimens were mounted on stubs, sputter-coated in gold, and kept in the desiccator with silica gel until observation in an SEM (Alves et al. 2013). The Electron Micrographs were obtained using Scanning Electron Microscope (SEM) of types LEO EVO 40 and TESCAN CLARA.

2.7. Statistical analysis

All data were collected and organized in spreadsheets in Microsoft Excel. All data were tested for normality and homogeneity before analysis of variance (ANOVA). Statistical analysis of variance (ANOVA) was performed using R software, and the averages were compared with the Scott Knott test served to determine statistically significant differences which were accepted on the 95% significance level ($P < 0.05$). The experiment was designed in complete randomized design (CRD) in six treatments and seven replications, each pot containing three plants. The test was repeated four times. Data from two severity experiments were analyzed together, AUDPC and growth promotion. For the other parameters, namely the microscopy images, the number of panicles and the production are taken from a single experiment.

3. RESULTS

3.1. Biocontrol effects of Bacillus sp. BMH on rice blast severity

Bacillus amyloliquefaciens BMH was efficient in reducing disease severity (Fig. 2A) after soil inoculation by 53.12%. The foliar prevention treatment (T5) 24 hours before challenge application compared to the control treatment by 50%. The maximum disease severity was 40% 28 DAI for blast positive control followed by the foliar mixture of bacteria-pathogen treatment (T4) that showed 25%. The significantly lowest severity was observed in soil inoculation with *Bacillus* treatment (T6) followed by the preventive control treatment (T5), with 20% and 18.8%, respectively (Fig 2A). Disease severity data over time was integrated into the area under the disease progress curve (AUDPC) (Fig. 2B). The AUDPC showed a significant difference between the index blast positive treatment (T2) with value 513.63 compared to 274.75; 221.46, and 195.48 in T4, T5, and T6, respectively (P-value < 0.027).

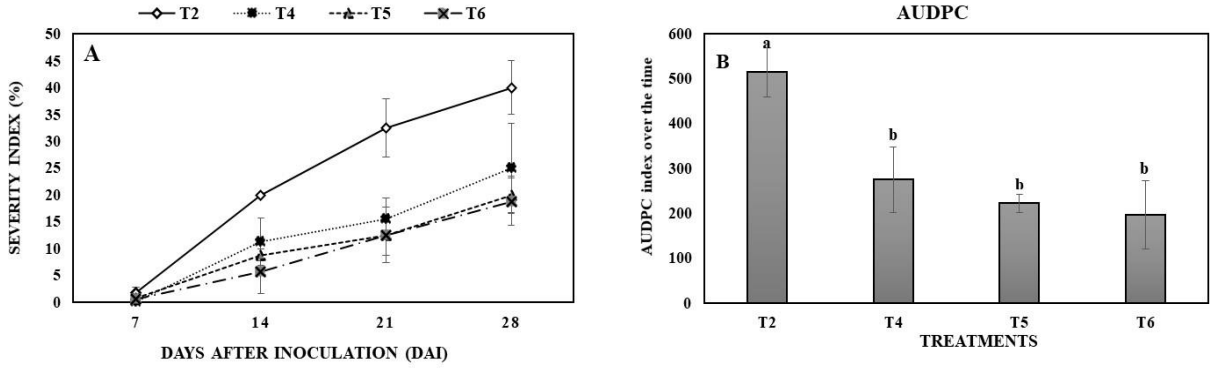

Figure 2- Disease progress curves (A) and area under disease progress index (AUDPC) in (B) in the biocontrol of *Pyricularia oryzae* with *Bacillus amyloliquefadiens* BMH under greenhouse conditions. Different letters indicate statistically significant differences among the treatment (Scott-Knott - $p < 0.05$). T1: Negative control (no pathogen); T2: Positive control (only the pathogen); T3: BMH positive (soil inoculation); T4: Mixture BMH- pathogen); T5: BMH and pathogen later; T6: BMH in soil and pathogen later.

3.2. Influence of *Bacillus amyloliquefaciens* in plant growth and grain weight

Soil inoculation with *Bacillus* (T3) before rice sowing had a significant effect on all plant heigh, tillers and panicle numbers, and weight of grains (Fig.3A-B). Rice plants were significantly higher in presence of BMH with 28.5 cm (T3) compared to the negative control with 25.7 cm (T1) DAS. Likewise, the number of tillers was significantly different with 12.6 for BMH (T3) against 9.1 for negative control (T1). We observed that BMH associated plants presented a darker green coloration and more vigor compared to the negative control plants. In the same way, the number of panicle and grain weight was significantly higher in presence of BMH (10.2 g) compared to the negative control treatment (7.1 g) (Fig. 3C) which showed its biofertilizer activity.

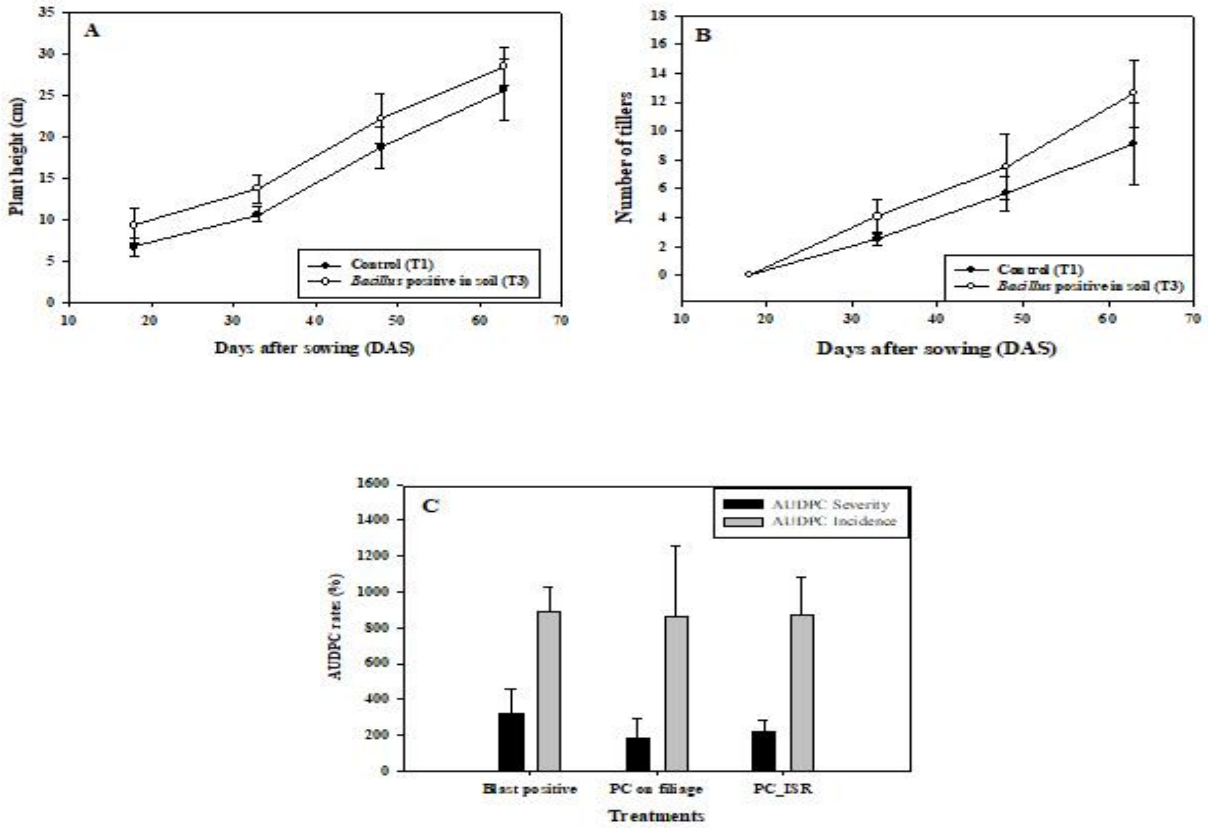

Figure 3- Effects of *Bacillus amyloliquefaciens* BMH in the growth promotion of rice plants and weight of grains. Rice plant height (A), number of tillers (B), number of panicles and grain weight (C). Different letters indicate statistically significant differences among the treatments (Scott-Knott - $p < 0.05$). Bars represent the standard error of each treatment.

3.3. Confocal Laser Microscope analysis in microorganisms-rice plant interaction

The confocal microscope revealed the root vascular colonization tissue by *B. amyloliquefaciens* BMH (Fig. 4DF) when the soil was inoculated with bacteria and showed the lowest severity compared to the control treatment. In the blast positive treatment (T2) and the mixture of *Pyricularia-Bacillus* treatment (T4), *P. oryzae* infected and colonized the rice leaves (Fig. 4CE). *Bacillus amyloliquefaciens* cells were also observed in green color in leaves veins in T4 (Fig. 4 E). We did not find any bacterial cells on the rice leaves surface or in the stomata in T5. Soil inoculation with *Bacillus* in treatment T6 asymptotically colonized rice root vascular tissues (Fig. 4 DF), which confirm its endophytic ability and is on rice cultivar BRS.MG Caçula.

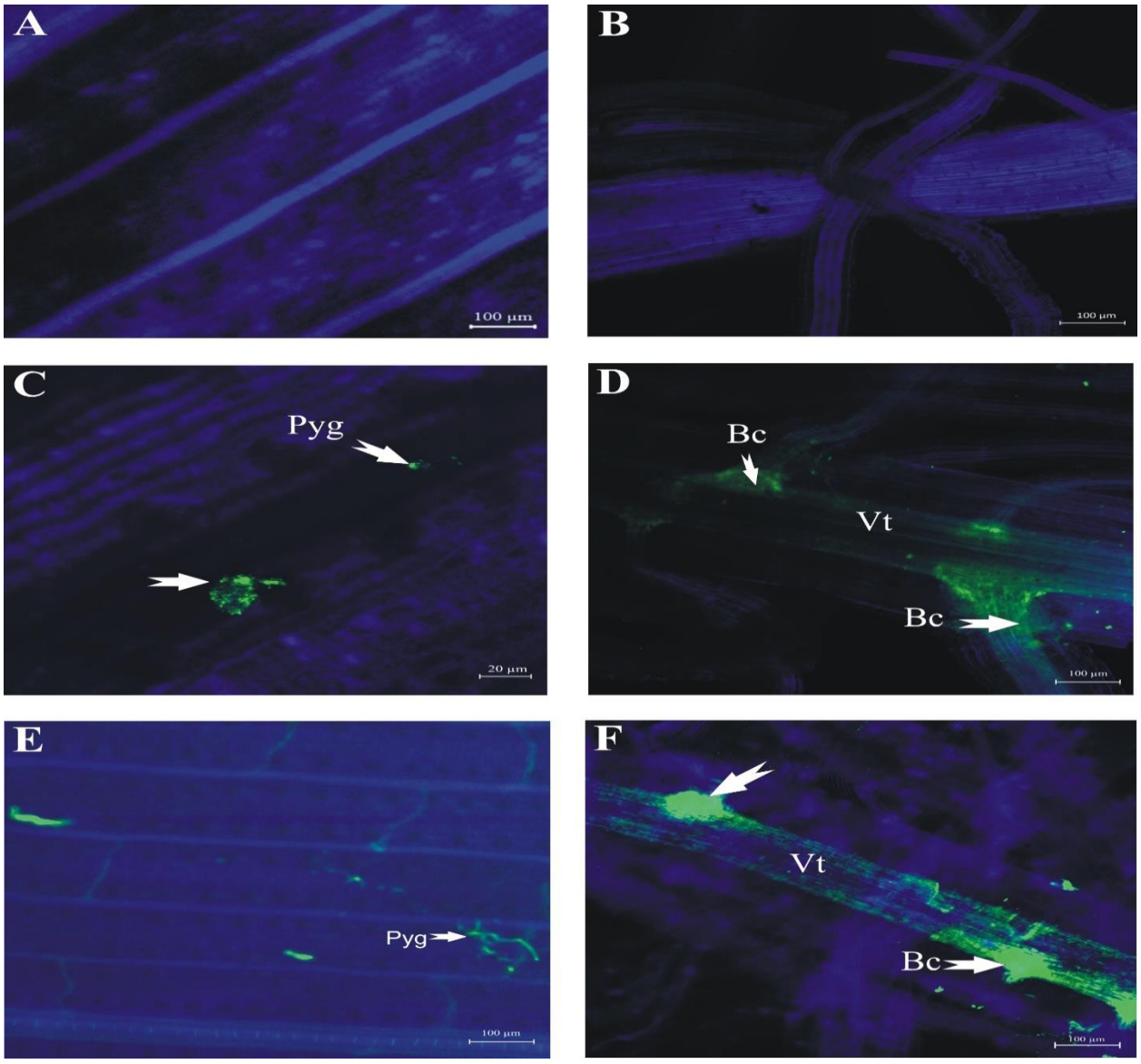

Figure 4 - Confocal microscope images of *Pyricularia oryzae*, bioagent *Bacillus amyloliquefaciens* BMH, and rice plant interaction. Colonization of the vascular bundles from rice cultivars BRS-Caçula at 22 days post-inoculation (dpi) of seeds for the root, and 3 days post-inoculation (dpi) of healthy rice leaves. A and B indicate the control treatment, leaf and root respectively, without pathogen. C and E the green mycelial indicate *P. oryzae* (Pyg) on leaf surface three dpi with Alexa Flour. D and F positive control treatment (soil inoculate with *Bacillus* sp.) with bright green indicate Bacteria colony (Bc) in vascular tissue (Vt) dyed with SYTO9 in the rice root.

3.4. Scanning Electron microscopy analysis in microorganisms-rice plant interaction

The Scanning Electron Micrograph showed that the control treatment (T1) is free from the attack of the pathogen on the leaf surface (Fig. 5A), and the colonization of the rice root vascular tissues by BMH (Fig. 5B). We observed the germinating conidia of *P. oryzae* on the rice leaf surface in blast positive (T2), in BMH preventive control (T5), and BMH soil inoculated (T6) treatments, respectively (Fig. 5CFG). We did not find *Pyricularia* conidia on the sample collected in treating T4. On another side, the bacterial colonies were observed in rice root vascular tissues of BMH soil inoculated treatment (T3) (Fig. 5D). Likewise, the destroyed leaf containing the bacterial cells in the mixture of *Pyricularia* and BMH treatment (T4) as well as BMH soil inoculated treatment (T6) was observed (Fig. 5EH). This analysis demonstrated the ability of *Bacillus* BMH to colonize rice root vascular tissues as observed in CLM analysis (Fig. 5EF) as well as leaf tissues, confirming its endophytic potential.

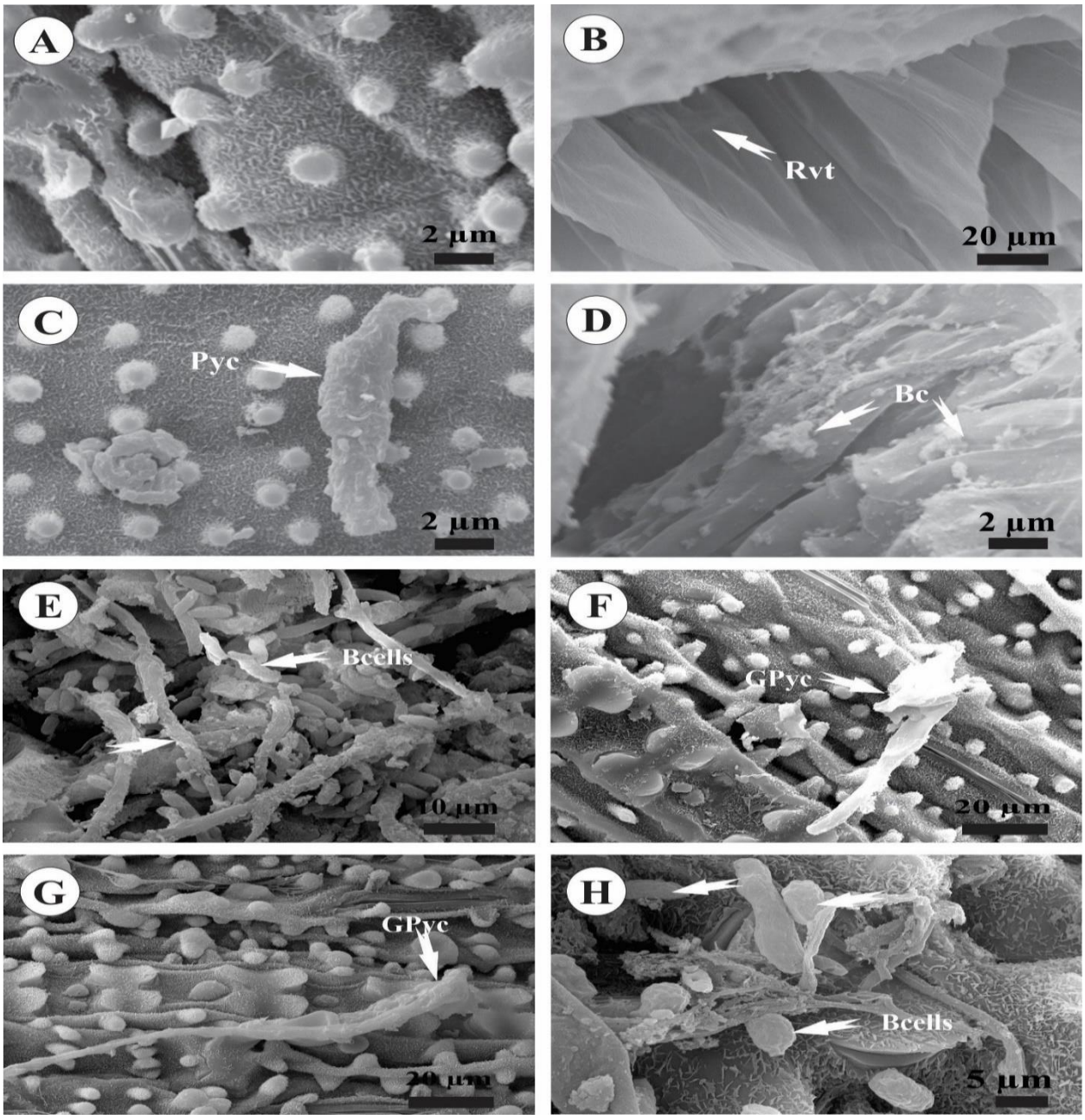

Figure 5- Scanning Electron Micrographs of *Pyricularia oryzae*, *Bacillus amyloliquefaciens* BMH, and rice plant interaction. Colonization of rice seedling of the cultivars BRS-Caçula at 21 days after inoculation (dai) of soil for root sample, and 3 days post-inoculation (dpi) of healthy rice leaves. (A, B) indicate the blast negative treatment (T1) of the rice leaf surface and root respectively. (C) represents rice leaf of blast positive treatment with *P. oryzae* conidia germinating (Pyc) on the leaf surface. (D) indicates the *Bacillus* positive treatment with bacteria colonies in rice root vascular tissues.

(E) represents the destroyed leaf containing the bacterial cells where the leaves were sprayed with the mixture of *Pyricularia* and BMH (T4). (F and G) indicate the germinated *Pyricularia* conidia (GPyc) on the leaf surface in T5 and T6, respectively. Finally, (H) indicate the destroyed leaf containing the bacterial cells in T6. The size bar in A, B, C, D, E, and F, are respectively 2, 20, 2, 2, 10, 20, 20, and 5 μm.

3.5. Rice resistance genes expression profile

The result of gene expression analyses showed that the rice OsALD1 gene was slightly expressed in only T5, with the relative expression values 0.01 at 48-hour post-inoculation (hpi) (Fig. 6A). The rice acidic pathogenesis-related (PR) protein (OsPR1a) was expressed in all *P. oryzae* treatments except the untreated T1. OsPR1a was more expressed in treatment T6 than other treatments T2, T4, and T5 with the relative expression values 0.38, 0.19, 0.7, and 0.1 respectively (Fig. 6B). We hypothesized that the OsPR1a expression was triggered by microbial inoculation (bacteria mainly because it is expressed in only pathogen's inoculated treatments. Concerning the rice basic PR (OsPR1b) gene, it was more expressed in blast positive T2 with 0.14 as the expression values, and slightly expressed in both T4 and T1 with the value 0.04 (Fig. 6C). In T6 in BMH soil inoculation, there was no expression of OsPR1b. Like the OsALD1 gene, we hypothesized that the application of *Pyricularia* and *Bacillus* mays downregulate the OsPR1b gene expression in this study. In the same way, a rice endo-1,3-β-glucanase gene (OsGLN) was more expressed in presence of BMH in T4 and T6 with the relative expression 0.49 and 0.42, compared to other treatments T1, T5, and T2 with 27, 0.04, and 0.01, respectively (Fig. 6D). The OsWRKY28 gene was more expressed in the mixture of BMH and *Pyricularia* treatment (T4) compared to others with the relative expression values 0.04, 0.02, and 0.01 for T4, T2, and T6, respectively (Fig. 6E). In comparison to T2, the OsWRKY28 gene was up-regulated in T4 and downregulated in the case of BMH soil inoculation in T6.

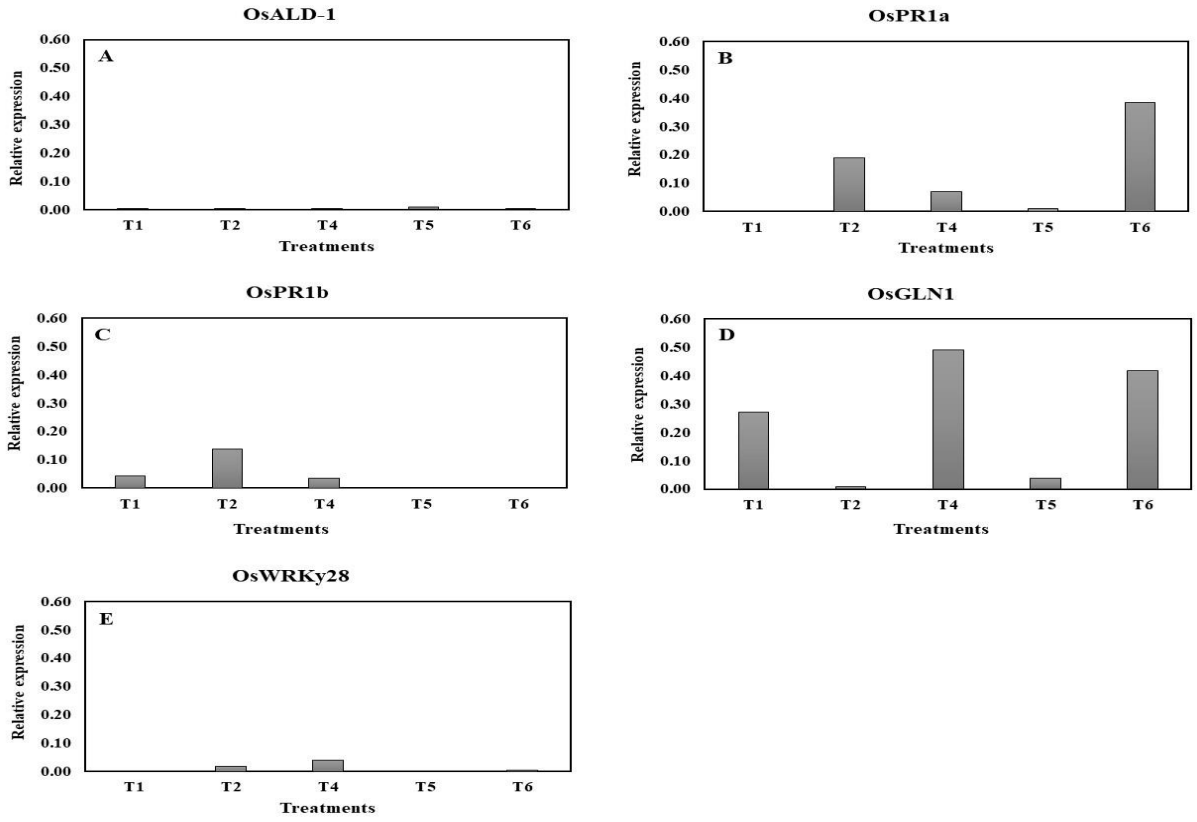

Figure 6- Relative expressions profiles of genes for rice resistance to blast disease. Each graph represents an expression of a single gene in different treatments OsALD-1, OsPR1a, OsPR1b, OsGLN1, and OsWRKy28 genes respectively. Sampling was carried out 48 hours post-inoculation (hpi) of the microorganisms on the leaves surface. Gene expressions were normalized based on the expression of the constitutive gene 7ubiq and shown as relative expressions to the corresponding values of non-inoculated leaves.

4. DISCUSSION

The use of the endophytic *Bacillus amyloliquefaciens* BMH successfully controlled rice blast disease, while improving plant growth, tillering, and grain yield. Research by He et al. (2019) showed that rice seeds soaked in the culture broth of *B. subtilis* before planting induced rice systemic resistance to the rice plant by inhibiting blast disease development over 70%. Also, three strains of *Bacillus* spp. have been reported to be effective against red rot disease caused by *Colletotrichum falcatum* by 45-49% in sugarcane by ISR in sugarcane plants (Hassan et al. 2012).

This research does not disagree with our study on the induction resistance to plants by the *Bacillus* genus. A field trial investigated by Gnanamanickam; Mew (1992), reported the ability of *Bacillus* sp. to decrease blast disease by 44- 46% in rice plants. In further research *Bacillus* spp. were able to activate growth and defense systems in the host plant in various environments and conditions (Cawoy et al. 2009). According to AFZAL et al. (2019) endophytic bacteria present themselves as a producer of stimulators of many bioactive metabolites such as flavonoids, peptides, alkaloids, phenolics, terpenoids, flagella, Quorum Sensing (QS), siderophore, not only but also phytohormones to protect their host plants, while improving its fitness. These findings corroborate the current study where *B. amyloliquefaciens* BMH protect the rice plant by antibiosis and stimulation of plant defense mechanism.

In this study, it was found that the plants inoculated with *Bacillus* BMH were higher than untreated ones, not only but also the number of tillers and panicles, and grain weight. Nowadays, plant growth-promoting rhizobacteria (PGPR) are being widely used as an alternative to chemical fungicides and fertilizers (Tobergte and Curtis 2013). Several previous studies related the role of *Bacillus* to stimulate directly biological nitrogen fixation, producing phytohormones such as auxins, cytokinins, and gibberellins, while induction of systemic resistance to the host plant (Van Loon 2007). Sessitsch et al. (2012) reported that the endophyte bacteria community that colonizes rice roots have a high potential for plant-growth promotion. According to Shafi et al. (2017) and Compant et al. (2005), the volatile compounds produced by *B. subtilis* play an important role in plant growth promotion and the activation of a plant defense mechanism by triggering systemic resistance. It had also been reported that *Bacillus* species have high efficiency in host root colonization and production of growth metabolites that result in improving crop yield (Khalid et al. 2004) as shown in this study. An investigation by Saranraj (2014) suggested that *Bacillus* is enhance plant growth promotion (PGPR) activity by the synthesis of auxin, cytokinin, and gibberellins metabolites. The finding of all these authors goes in the same direction as ours in severity as well as PGPR evaluations. For Vaikuntapu et al. (2014) PGPR biocontrol activity is dependent on the host crop, the climate, and BCA strains.

Using CLM and SCM demonstrate the capacity of *Bacillus* BMH to colonize rice cultivar BRSMG Caçula roots and leaves that may play a key role in rice plant fitness, growth, and

activation of a plant defense mechanism. Rhizobacteria are bacteria from the rhizosphere that can colonize the developing root system in the presence of competing soil microflora (KLOEPPER et al. 1999). Inside the plant, endophytic bacteria colonize the plant systematically by migrating through the vascular system, the apoplast or remain localized in the root cortex or the xylem (Hurek et al. 1994). Subsequently, the study of Fan et al. (2012) demonstrated the ability of *B. amyloliquefaciens* FZB42 to colonize all parts of different plants as demonstrated by the microscopic analysis of this study.

Jung et al. (2016) reported that the ALD1-LIKE gene of rice (OsALD) was strongly transcribed in the infected leaves of plants by *P. oryzae*. In this study, the OsALD1 gene was expressed only in *Bacillu*s BMH preventive inoculation on rice leaves surface 24 hours before the challenging pathogen. We hypothesized that the expression of the OsALD1 gene could depend on the time of sample collection after inoculation with BCA and pathogen, and the age of the plant as suggested by Ponciano et al. (2007).

Research from Ponciano et al. (2007) has shown that transcription of PR defense gene expression (OsPR1a, OsPR1b) is correlated with both *Xanthomonas oryzae* pv *oryzae* virulence gene (Xa21) and the Avr factor for the highest levels of induction of resistance. The rice adult stage leaves could be more competent to express OsPR1 genes against Xa21 in the incompatible interactions at three days (72 HPI) (PONCIANO et al. 2007). In our study, OsPR1b was highly downregulated in comparison to control and blast positive treatments at two days (48 HPI). Whereas OsPR1a was expressed either by blast positive, and *Bacillus* inoculated treatments, indicating that its expression is dependent on the attack by pathogen as reported by Wu et al. (2011). Although the precise function of any PR1 protein is not known, it is evident that the rice PR1a gene was upregulated in *Bacillus* BMH association with rice plant in soil inoculation. The finding by

Plants, β -1,3-glucanases which is thought to play a key role in plant normal growth and development, has been referred to as PR proteins, playing a direct role in defenses against fungi by hydrolyzing fungal cell walls and exhibiting antifungal activity (AKIYAMA et al. 2004; WAN et al. 2011). β -1,3-Glucanases genes have been identified in a variety of plants including rice, that catalyze the hydrolysis of β -1,3-glucans, which are found in the cell walls of various plant tissues and fungal pathogens (DIXON et al. 1996). Akiyama; Pillai, (2001) reported that glutathione S-

transferase (GST)-OsGLN1 recombinant protein rapidly hydrolyzed the cell wall b-glucans of *P. oryzae*. In this study, the expression of the OsGLN1 gene was upregulated with the mixture of *Bacillus* BMH on foliage and its soil inoculated treatments, confirming its ability to induce resistance and promote rice plant growth. It has been suggested that the expression of OsGLN1 could also be up-regulated by drought stress and ABA treatment and the accumulation of its transcript is more in the roots of rice seedlings (AKIYAMA; PILLAI, 2001).

A single WRKY gene would often respond to multiple stressors, and their proteins could participate in the regulation of multiple disparate processes as negative or positive regulators (Chen et al. 2012). According to Chujo et al. (2013), the overexpression of OsWRKY28 in rice plants resulted in increased susceptibility to *M. oryzae*. They strongly suggested that OsWRKY28 is a negative regulator of basal defense responses against *P. oryzae* and acts as a modulator to maintain responses at an appropriate level by attenuating the activation of expression levels of genes linked to defense (CHUJO et al. 2013). In our study, this gene was up-regulated in the mixture of BCA and pathogen inoculation on the foliage and downregulated in the case of BMH soil inoculation in comparison to the blast positive treatment.

5. CONCLUSION

In general, our results demonstrated the endophytic *Bacillus* BMH ability to control rice blast disease, while improving plant growth, tillering, and grain weight. These results suggested that *B. amyloliquefaciens* inoculation on the seed may be a good way to control *P. oryzae* through the mechanisms of antibiosis and induction systemic resistance while improving plant growth and yield through its biofertilizers activities. We also demonstrated that *Bacillus* BMH is a good endophyte of rice plant which could play a key role as a bioagent in the Sahel region of West Africa when applied in seeds drenching. The genes expression on plants can be improved by taking into account the developmental stage of rice and the organ to be sampled. Despite this, it is better to test its effectiveness in natural conditions where with multiple factors.

6. ACKNOWLEDGMENTS

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CHAPTER II- PUBLISHED ARTICLE IN THE BIOLOGICAL CONTROL-JOURNALS OF ELSEVIER

Control of blast disease caused by *Pyricularia oryzae* with *Epicoccum nigrum* and microscopic studies of their interaction with rice plants under greenhouse conditions

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ABSTRACT

Rice (*Oryza sativa* L.) feeds around half of humanity mainly in Asia, South America, and Africa. The filamentous, ascomycete fungus *Pyricularia oryzae* Cavara (syn: *Magnaporthe oryzae* B. Couch) hemibiotrophic phytopathogen is the causal agent of the most devastating disease, blast on rice. The fungus *E. nigrum* is ubiquitous resistant in adverse conditions and used as a biological control agent (BCA) against numerous phytopathogenic fungi in the function of its ability to

produce many secondary metabolites. This study aimed to evaluate the efficiency of an isolate of *E. nigrum* on rice blast disease suppression, as well as rice growth promotion and productivity of the cultivar BRS.MG Caçula, in pot assay under greenhouse conditions, and evaluate the interaction between plants and microorganisms, using microscopic approaching. Rice leaf blast disease incidence and severity were evaluated on seeds and plants, which were treated with *E. nigrum* and *P. oryzae* isolate IA25, and compared with the control treatment. Foliar spraying and coating of the rice seed with *E. nigrum* significantly reduced the incidence rate of rice blast from 31.25% to 41.76%, respectively, compared to the control treatment. Likewise, the severity the rate was reduced from 34.92% to 54.51%. In-plant growth, it was found that seed-soaking with *Epicoccum* provided plant fitness and increases the number of tillers to 8.34 against 5.95 for the control situation. The Epifluorescence Microscopy (EFM) and Scanning Electron Microscopy (SEM) showed the colonization of rice root by endophytic fungus *E. nigrum*. The greenhouse experiments confirmed that *E. nigrum* significantly decreased rice leaf blast disease incidence and severity rate while improving plant tillering.

Keywords: *P. oryzae*, incidence and severity, biocontrol, *E. nigrum*, plant growth promotion, microscopy.

1. INTRODUCTION

Rice (*Oryza sativa* L.) is the major staple food for approximately half of the world's population (Srivastava et al. 2017). It's primarily for direct human consumption, remains an important staple food in Asia, Africa, Latin America, and the Caribbean (Koizumi and Furuhashi 2020). Rice occupies an important place in the food diet of Africans and it represents more than 25% of the total cereals consumed following the corn (Villar & Bauer, 2019).

On rice, more than 70 different diseases caused by fungi, bacteria, viruses, and nematodes have been listed (Huijuan Zhang et al. 2009). Among them, there is the filamentous, heterothallic ascomycete fungus *Pyricularia oryzae* Cavara (syn. *Magnaporthe oryzae* B. Couch) is a seed-borne and airborne plant pathogen that causes the most devastating blast disease of rice (Gladieux et al., 2018). It is a cosmopolitan pathogen detrimental in lowland and upland rice, and it can infect all tissues of rice including the root (Nguyen et al., 2016; Zhang et al. 2016). *Pyricularia* is a multi-

host pathogen that infects more than 50 other species of cereals and grasses (Gladieux et al., 2018; He et al., 2019), and it causes enormous economic and important disease (Howard and Valent, 1996). The annual losses of rice caused by *P. oryzae* vary from 10% to 30% of the production, or even 100% with the very sensitive cultivars (Agrios, 2005; Raveloson *et* al, 2013; Spence et al. 2014).

Blast disease control strategies adopted include cultural strategies, genetic resistance, and chemical fungicides are effective and have long been used to reduce its severity (Pooja et Katoch, 2014; TeBeest et al. 2015). However, the use of resistant cultivars and chemical fungicides although efficient have shown their limits, because of the pathogen's ability to evolve to overcome the resistance gene and the appearance of resistant strains, in addition to the fungicides hazards on the environmental (Nguyen et al. 2016; Raveloson et al. 2013). Therefore, consumer awareness of the harmful effects of agrochemicals on human health and the environment shows that alternative, more integrated, and sustainable strategies are needed in crop disease management practices. To this end, the use of bioagents, due to their long-lasting mechanisms for controlling pathogens and promoting plant growth, is increasingly widespread in the agricultural industry (Shah et al. 2021; Dubey et al. 2015). This strategy is a promising and environmentally friendly approach that can be used alone or in combination with other approaches in Integrated Pest Management (IPM) (Dubey et al. 2015).

The endophytic fungus *E. nigrum Link (syn. E. purpurascens Ehrenb. ex Schlecht.)* is ubiquitous, found in air, soil, and on decaying vegetation (Braga et al. 2018). It is used as a biocontrol agent against numerous phytopathogenic fungi and for its ability to produce many secondary metabolites, such as antioxidant, and antimicrobial compounds (Pascual, et al. 2000; Fávaro, et al. 2011). According to Li et al. (2013), the production of the antifungal compound, the production of large spores with large amounts of stored nutrients, and the increased ability to survive unfavorable climatic conditions make *Epicoccum* an attractive candidate as a bioagent. Species of *Epicoccum* are famous for their application in the biocontrol of several phytopathogens their capability of producing various biologically active compounds with medical applications as antioxidant, antimicrobial, and anticancer agents (Elkhateeb and Daba 2019). Some studies suggested that *E. nigrum* is considered as epiphytic and endophytic fungus (Fávaro et al. 2012),

possessing melanized conidia which increases its tolerance to ultraviolet (UV) rays and can colonize the phylloplane (De Cal et al. 2019). It is considered a promising source of a wide variety of vital metabolites such as alkaloids, flavonoids, phenols, steroids, and terpenoids (Nisa et al. 2015). *E. nigrum* had been used to control *Pythium irregulare* (Koutb et al. 2018), *P. debaryanum and P. ultimum* (Hashem and Ali 2010), *Alternaria solani* (Nafady et al. 2017), *Phytophthora infestans* (Li et al. 2013), *Monilinia* spp (Larena and Melgarejo, 2009). *E. nigrum* is mainly associated with the primary decomposition of plant tissues, has been described as a weak plant pathogen of *Cucumis melo* (Braga et al. 2018).

To better understand the interaction between plant, pathogen, and bioagent, histological studies are important to provide accurate information about the development and tissue colonization by BCA as well as challenged pathogen during the infection process. That is why we used Scanning Electron Microscopy (SEM), and Epi-Fluorescence Microscopy (EFM) to observe these interactions. Thus, the current study seeks (i) to evaluate the efficiency of an isolate of *E. nigrum* in rice blast disease suppression, (ii) in the rice cultivar BRSMG Caçula growth promotion and productivity, (iii) as well as analyze the interaction between plants and microorganisms, using microscopic approaches, and pot assay under greenhouse conditions. In this study, the intention is to develop management strategies to minimize the use of fungicides and chemical fertilizers, and therefore their environmental impact by using beneficial endophytic *E. nigrum* to improve the health of rice plants against blast disease, plant growth, and yields to meet food demand.

2. MATERIAL AND METHODS

2.1. Plant material and microorganisms

The rice (*Oryza sativa* L.) variety, BRS.MG Caçula is considered very susceptible to *P. oryzae* in edaphoclimatic conditions from Minas Gerais state, Brazil, which was used in this study. *P. oryzae* strain IA25 (BOTELHO, 2019 unpublished data) which is considered as one of the most virulent strains in Minas Gerais state, has been obtained from the Department of Plant Science of UFLA, Brazil. The *P. oryzae* was reactivated on potato dextrose agar (PDA) and for conidia mass

production at 25± 2°C. *Epicoccum* strain used in the experiments was obtained from the Molecular Phytopathology Laboratory of the Phytopathology Department (DFP). It had been isolated from Vitis *vinifera* (grapevine) at the Epamig Anthology experimental station in the municipality of Caldas, Minas Gerais, Brazil.

2.2. Microorganisms mass production, plant growth conditions, and inoculation

Pyricularia strains IA25 was multiplied in Petri dishes containing Potato-dextrose-agar (PDA) or Oatmeal Agar (OA) medium supplemented with antibiotics (streptomycin +chloramphenicol 0.05g) and incubated at 25 ± 2ºC (with 12-hour photoperiod) (Silva et al. 2017). After 15 days of incubation, the sterilized rice seeds were distributed on the colony of *P. oryzae* (20 seeds for Petri dish) and incubated in the refrigerator at 25 ± 2ºC (with 12-hour photoperiod) for 24 hours, before soaking the seeds with mycelium and conidia suspension on sowing moment. To eliminate surface contaminating microbes, the rice seeds were thoroughly surface treated with sodium hypochlorite 1% for 1mn and 70% ethanol for 1minute each, followed by three rinsed steps in sterile water before dried under room temperature. Rice seeds were sowed in a pot filled with 800 g of the mixture of sterile sand (1/5) and substrate soil (4/5) was used for the rice seedlings before being kept in a greenhouse at 30°C temperature maximum. This substrate named Trosptrato® HA Hortaliças was composed of Pinus Bark, Vermiculite, PG Mix 14.16.18, Potassium Nitrate, Simple Superphosphate, and Peat. For rice seedling inoculation, the Petri dishes containing *P. oryzae* sporulated were carefully washed with water containing Tween 20 and gelatin [1% (wt/vol)] according to Araújo et al. (2019). The conidial suspension was calibrated with a hemocytometer to obtain a concentration of 1×10^5 conidia/mL. On the other side, as the previous antagonist, *E. nigrum* was grown in the Petri dishes containing PDA and OA medium, incubated for 20 days in the refrigerator at 25 ± 2ºC (with 12-hour photoperiod). The conidial suspension was prepared by scraping the fragments of the mycelium and conidia with a paintbrush. Mycelium and conidia suspension was used to soak rice seeds before sowing like the previous. The final conidial suspension was adjusted to the 1×10^6 conidia mL^{-1} for rice seedling leaf inoculation and mixed with 15 µM of glucose for its growth. After that, all the treatments were subjected to respective

artificial inoculation with *P. oryzae* and candidate antagonists (Table 4).

The fertilizer NPK 20-5-20 was applied at the recommended rate (90 kg. ha-1), while nitrogen up to recommended rate (80 kg. ha-1) was applied twice (40 kg. ha-1 at 15 days after sowing and 40 kg. ha-1at the stage of tillering at 45 days). After spraying pathogen and BCA, rice seedlings were incubated in a growth chamber at 80 to 100% relative humidity at 25°C in the dark for 24 hours to stimulate the infection. Subsequently, the pots were transferred to the greenhouse.

The experiment was arranged in a completely randomized design (CRD) with seven replications and seven treatments. Two replicates per treatment were used for microscopy studies and five replicates for disease incidence, severity, plant growth promotion, and productivity assessment.

Table 1. Treatments put in competition for the control of *Pyricularia oryzae* with *Epicoccum nigrum.*

Treatments	**Designation**	**Inoculation time**	**Plant growth stage**
T1	Blast negative treatment	18 days after sowing (DAS) with water	Beginning of tillering
T2	Blast positive treatment on seed	18 DAS with *P. oryzae*	Seed distributed on *P. oryzae* colony (24h), soak with a conidia suspension
T3	*Epicoccum* positive treatment	*E. nigrum* in sowing day	Seed inoculation before sowing
T4	Control in seed germination	*A mixture* of *Epicoccum* x *P. oryzae* in sowing day	Seed soaking with the mixture of conidium
T5	Induction of Systemic Resistance (ISR)	*Epicoccum* sp. before sowing and 18 DAS with *P. oryzae*	Seed soaking and the beginning of tillering
T6	Foliar preventive control	18 DAS *E. nigrum* first, and *P. oryzae* 24 hours after	Beginning of tillering
T7	Blast positive treatment on foliar	18 DAS with *P. oryzae*	Beginning of tillering

2.3. Sample preparation for microorganisms-plant interactions observation in Epi-Fluorescence Microscopy (EFM)

Samples of roots and leaves of rice inoculated with microorganisms (pathogens or antagonists) and cut into pieces of 4x4 mm had been prepared according to Moreira et al. (2019). The leaf fragments and roots were placed within Elisa plates well containing KOH 10%, following its incubation at 10°C for 4 days in the darkness. Samples were then washed with PBS and replaced with a new KOH 10% solution for 4 days again. It was thereafter washed with PBS and transferred in the Elisa 96 well plates with clarifying mix [Urea 6M, Glycerol 30% (v/v) and Tween 20 0.05% (v/v)]. After 4 days in the clarifying mix at 10°C in the darkness, washed with PBS, and incubate in a new clarifying mix for 4 days. Thereafter, washed again with PBS before adding 100 µL Alexa488–WGA dye 10 µg. mL^{-1} to see the root or leaf surfaces. Elisa plates were wrapped with aluminum foil and kept in a vacuum for 1 h. Then, 80 µL of Calcofluor dye 0.01 mg. mL-1 were added and incubated for 10 min in darkness to see the fungus. A previously cleaned large coverslip was put on an inverted CLM stage. Then, the tissues fragments were put above the coverslip with the region of interest facing down and a glass piece above the sample (to minimize the irregular topography of the sample). In these assays, the images were obtained with 3D reconstruction using the Epi-Fluorescence Microscopy (EFM).

2.4. Scanning Electron microscopy (SEM) sample preparation

The collection of rice plant leaves, roots was done 21 DAS or 3 days after BCA or pathogen inoculation (Table 1). The parts of interest were selected and cut into pieces of a maximum of 2 cm inside and long by up to 2 cm high fixed in Karnovsky fixative for 24 hours. The samples were then washed with cacodylate buffer and transferred to sequential dehydration in 25%, 50%, 70%, 90% acetone for 10 min each, and 100% 3 times for 10 min each. Then, samples were transferred to the critical point dryer to complete the drying process with carbon dioxide as the transition fluid before being mounted on aluminum while taking care to keep the area to be observed upwards. Thereafter, takes place the sputter-coated in gold, and is kept in the desiccator with silica gel until

observation in an SEM (Alves et al. 2013).

2.5. Sequencing of ITS, RPB2, TUB and LSU rRNA gene amplified from the total DNA extracted

Extraction and purification of nucleic acids are the first steps in most studies of molecular biology and all recombinant DNA techniques. Removed the mycelium and conidium from the Petri dishes and smoked it with liquid nitrogen and put in the Eppendorf and added 750 µl of CTAB (Cetyltrimethylammonium bromide) (Leslie and Summerell 2006). Added 7.5 µl of β-mercaptoethanol and vortex for 2 min. Then put in the ban marry for 10mn at 65°C and vortex again for 2 min. Again, in the ban marry for 20 nm at the same temperature. Added to the supernatant 700 µl of chlorophyll (chloroform + alcohol + isopropyl 24: l) and vortex for 1 min. Then centrifugate at 13000 rpm for 10 min. Transfer 500 µl of the supernatant in another new tube and add 500 µl of isopropanol. Also, added 50 µl of diacetate of sodium. Then, centrifugate it again before removing the supernatant from the pellet in the Eppendorf and adding 500 µl of ethanol 70%. Centrifugate it at the same rpm for 5mn before eliminating ethanol from the pellet in the Eppendorf. Dry the pellet for 20 min and add 50 µl of buffer TE before keeping it in the refrigerator at 4°C. Account the pellet on nanodrop Lite before keeping it in the refrigerator at – 20°C. A part of the ITS, RPB2, TUB, and LSU genes was amplified and sequencing was conducted by the ACT ACTGene Molecular Analyzer DNA sequencing services (Brazilian company), using the PCR primers for the four genes (Appendix C).

In phylogenetic analyses, the appropriate taxa for the analyses were initially selected following BLAST searches of GenBank (http://www.ncbi.nlm.nih.gov/). The sequence datasets were compared with similar sequences from strains of other species in the *Epicoccum* species already available in GenBank and mentioned by Chen et al. (2015), and Jayasiri et al. (2017). The obtained sequence data were aligned with 11 reference sequences that were obtained from public databases (Table 5). The phylogenetic analyses were done for each data set separately, as well as with a combined alignment consisting of the four ITS, RPB2, β-TUB, and LSU regions. Using the Molecular Evolutionary Genetics Analysis (MEGA) software approach we built automatically the

phylogenetic tree and the evolutionary history was inferred using the Maximum Parsimony method (Kumar et al. 2018).

Table 2. *Epicoccum s*pp. used for phylogenetic analysis and their accession numbers.

Species	Culture accession number	GenBank accession numbers			
		ITS	LSU	rpb2	β-TUB2
Epicoccum nigrum	CBS 125.82	FJ426995.1	GU237974.1	KT389631.1	FJ427106.1
Epicoccum nigrum	CBS 173.73	MH860655.1	GU237975.1	KT389632.1	FJ427107.1
Epicoccum brasiliense	CBS 120105	GU237760.1	GU238049.1	KT389627.1	GU237588.1
Epicoccum draconis	CBS 186.83	GU237795.1	GU238070.1	KT389628.1	GU237607.1
Epicoccum henningsii	CBS 104.80	GU237731.1	GU238081.1	KT389629.1	GU237612.1
Epicoccum huancayense	CBS 105.80	MH861244.1	GU238084.1	KT389630.1	GU237615.1
Epicoccum pimprinum	PD 77/1028	FJ427050.1	GU237977.1	KT389633.1	FJ427160.1
Epicoccum plurivorum	CBS 558.81	MH861377.1	GU238132.1	KT389634.1	GU237647.1
Epicoccum sorghinum	CBS 179.80	FJ427067.1	GU237978.1	KT389635.1	FJ427173.1
Epicoccum sorghinum	CBS 627.68	FJ427072.1	GU237979.1	KT389636.1	FJ427178.1
Didymella anserina	CBS 253.80	KT389498.1	KT389715.1	KT389595.1	KT389795.1
Epicoccum nigrum (V. vinifera)	**OL652883**	**MZ577607.1**	**OL652883.1**	**OL677474**	**OM161959**
Epicoccum nigrum (Z. mays)	OL652884	MZ577608.1	OL652884.1	OL677475	OM161960

2.6. Assessment of blast disease, growth attributes and yield methods

To determine the influence of *E. nigrum* in leaf blast disease severity suppression, the blast was evaluated onto three leaves of each three plants per pot, recorded at 7 days post-inoculation (DPI) and every 7 days until severity stability or leaves senescence. The disease progress was scored on each leaf using visual quantification based on a diagrammatic scale of 0–9 developed by the International Rice Research Institute (IRRI, 2013). The reduction in leaf blast severity (LBS %)

$$\mathrm{AUDPC} = \sum_{i}^{n-1} \left| \frac{(yi + yi + 1)}{2} \right| (\mathrm{ti} + 1 - \mathrm{ti})$$

will be calculated relative to the severity in the inoculated control (100 − (severity of the treatment × 100 / severity of the control)). The disease score data were also converted to the area under the disease progress curve (AUDPC) according to the formula described by Madden et al. (2007).
where;
n = number of evaluations; y = disease intensity; t = is the time when assessing the intensity of the disease; (yi + yi + 1) = is the average height of the rectangle between the points yi and yi + 1; (ti + 1 + ti) = is the difference of the base of the rectangle between the points ti + 1 and ti, the time interval between disease assessment.

In-plant growth promotion assessment, the heights of the plants were measured and the number of tillers per plant was counted 18 days after sowing (DAS) and every 15 days until the number of tillers stability. Concerning the productivity, the number of hearts was counted per plant at harvest and the yield was measured after drying.

2.7. Statistical analysis

All data were collected and prepared in Microsoft Excel. All data were tested for normality and homogeneity before analysis of variance (ANOVA). Statistical analysis of variance (ANOVA) was performed using R software, and the averages were compared with the Scott Knott test served to determine statistically significant differences which were accepted on the 95% significance level ($P < 0.05$).

3. RESULTS

3.1. Effect of *E. nigrum* on rice blast disease incidence and severity suppression

The analysis of disease incidence and severity showed significant differences between treatments put in the competition. The result of the evaluation of leaf blast incidence and severity suggested that seed soaking with *E. nigrum* reduced disease incidence by 41.76%. Whereas, in preventive foliar treatment, *E. nigrum* decreased disease incidence by 31.25% in the last assessment in 28 days after inoculation (DAI) (Fig. 1A). Concerning the rice blast disease severity, it was

found 54.51% of disease severity reduction in seed soaking with *E. nigrum*, and 34.92% for foliar treated with *E. nigrum* in 28 DAI (Fig. 1B) below. The disease incidence data over time integrated into the area under the disease progress curve (AUDPC), showed no significant difference between the rates with 612.5 for (T6), 456.95 for (T5), and 865.28 for control (T7). In the disease severity, the rates 176.11; 109.66, respectively were observed with the preventive foliar treatment (T6) and the seeds soaking (T5), compared to the control treatment with 410.6 (T7) (Fig. 1C).

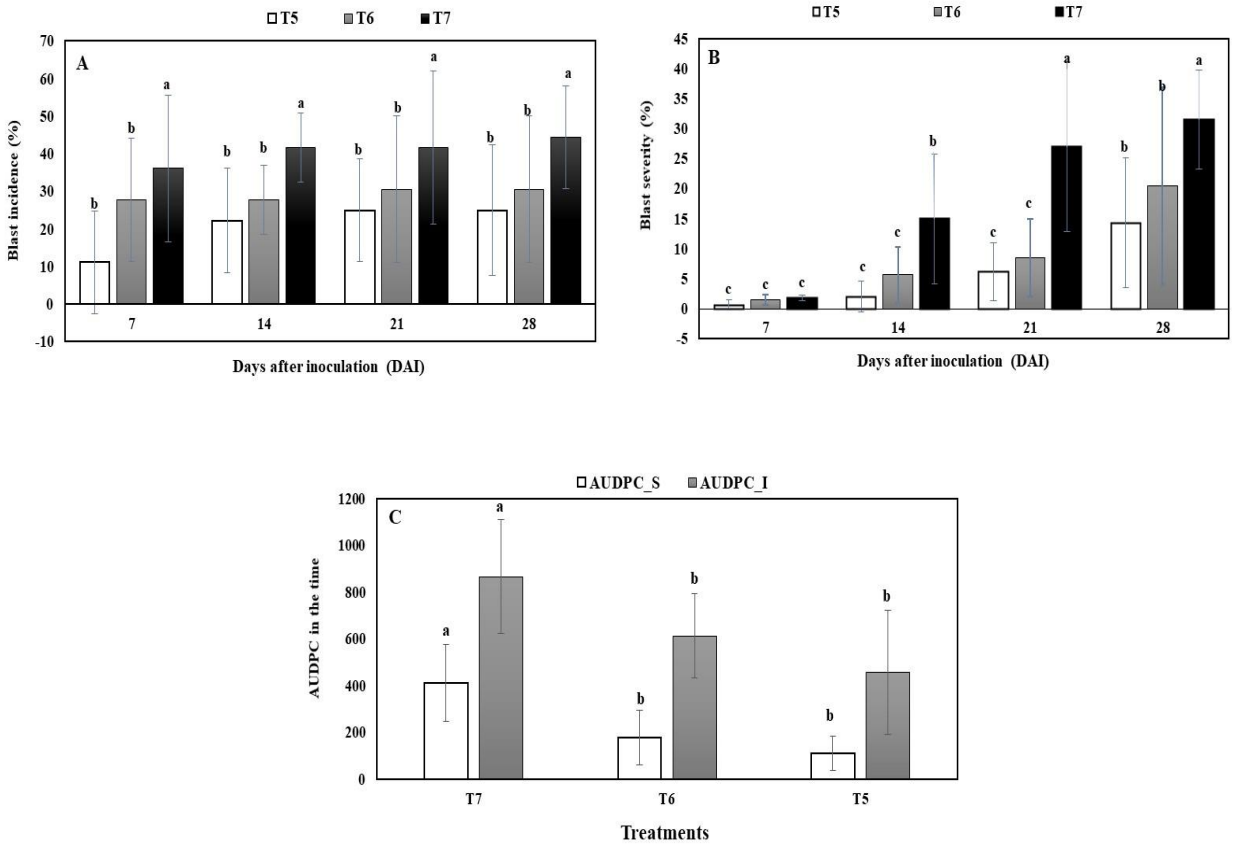

Figure 1- Effects of *Epicoccum nigrum* in rice blast disease incidence and severity. (A) disease incidence percentage in the function of days after inoculation (DAI), (B) disease severity percentage in the function of days after inoculation (DAI), on rice cultivar, BRS.MG Caçula inoculated with *P. oryzae* with P-value 0.003 and 0.000 respectively. In (C), AUDPCi for incidence and AUDPCs for the severity with P-value respectively 0.031 and 0.001. Each data point represents the mean ± standard deviation. Different letters indicate statistically significant differences among the studied treatment ($p < 0.05$), while the same letter indicates no significant differences among them, using the Scott-Knott test. Bars represent the standard error for each treatment mean.

3.2. Influence of *E. nigrum* on plant growth and tillering

In the growth promotion of the rice plant, there was no significant difference between treatments in plant height with P-value = 0.19 (Fig. 2A). However, it was observed that the rice plants of the seeds soaking with *Epicoccum* (T3) at 18 and 32 DAS were longer than those of the control treatment (T1), but the opposite was observed at the end. Whereas, the seeds treating *E. nigrum* before sowing enhanced rice tiller number, observed 46 days after sowing with significant differences (P-value = 0.018) between treatments with 8.34 for *E. nigrum* seeds soaked (T3) compared to 5.95 for untreated (T1) (Fig. 2B). With evidence, we hypothesize that *E. nigrum* would act as a growth regulator by decreasing plant height while increasing the number of tillers. Furthermore, it was noticed that the rice plants from *Epicoccum* seeds-soaked treatment (T3) retained the dark greenery even at the ripening stage, compared to the untreated (T1) (Fig. 1B in Appendix B).

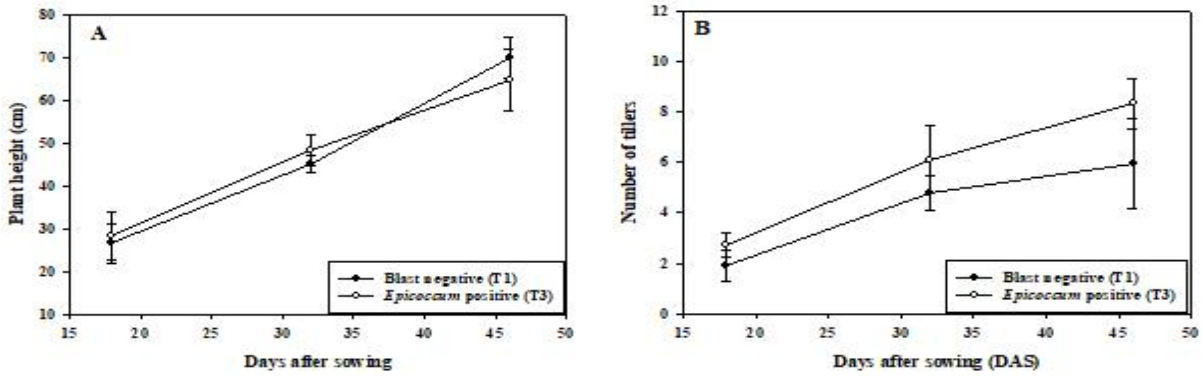

Figure 2- Influence of *Epicoccum nigrum* in the rice plant growth promotion. **(**D**)** represent rice plant height in centimeters in the function of days after sowing (DAS), and (E**)** plant tillering in the function of days after sowing (DAS). The bars represent the standard error of the mean. Different letters indicate statistically significant differences among the studied treatment ($p < 0.05$), while the same letter indicates no significant differences among them, using the Scott-Knott test. Bars represent the standard error for each treatment mean.

3.3. Influence of *E. nigrum* on rice plant number of panicle and grain weight

The number of rice plant panicles was not significantly different ($P < 0.05$) when the seeds is treated with *E. nigrum* (T3 and T4) with 9.33 and 8.1 per plant respectively, compared to the blast negative treatment (T1) with 6.66 per plant (Fig. 3A). However, although the seeds were inoculated with the biocontrol agent in T5 treatment, the number of panicles was significantly smaller than the others seed inoculated treatments above with 5.33. On the contrary, the higher values of yield were observed with (T3 and T4) with 6.78 g and 5.79 g per plant compared to blast negative treatment with 4.85 g per plant.

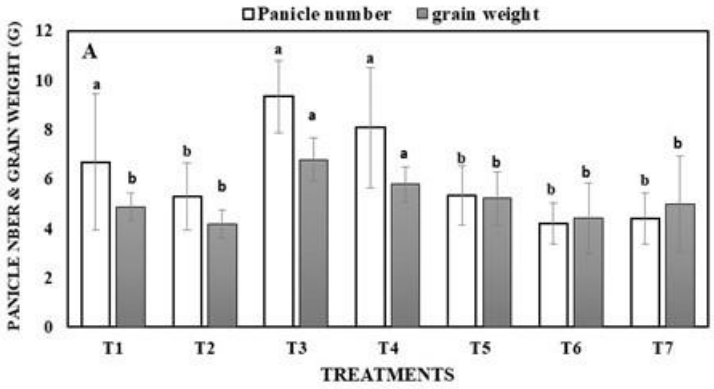

Figure 3- Influence of *Epicoccum nigrum* on the rice plant panicle number and grain weight. *Different letters indicate statistically significant differences among the studied treatment ($p < 0.05$), while the same letter indicates no significant differences among them, using Scott-Knott (P-value = 0.000 and 0.019 respectively, for panicle number and grain weight) significant different test. The bars represent the standard error of each treatment mean.*

3.4. Epi-Fluorescence Microscopy (EFM) analysis

For this image, it was observed that the surface of the leaves and roots of the blast negative treatment (T1) were free from any colonization (Fig. 4AB). In the blast positive treatment (T2) (Fig. 4C), *Pyricularia* conidia germinating was observable on the leaf surface. The EFM images showed that the seeds soaked with the biological control agent *E. nigrum* (T3) provided good colonization of the rice roots (Fig. 4B and C). We provide evidence-based on the EFM analysis

that *E. nigrum* is the endophytic fungi that colonized rice roots. The image of rice seeds inoculated with the mixture of *E. nigrum* and *P. oryzae* (T4) allowed observing the rice roots colonization by the mycelium of both fungi, where *Epicoccum* mycelium transcended those of *P. oryzae* (Fig. 4D).

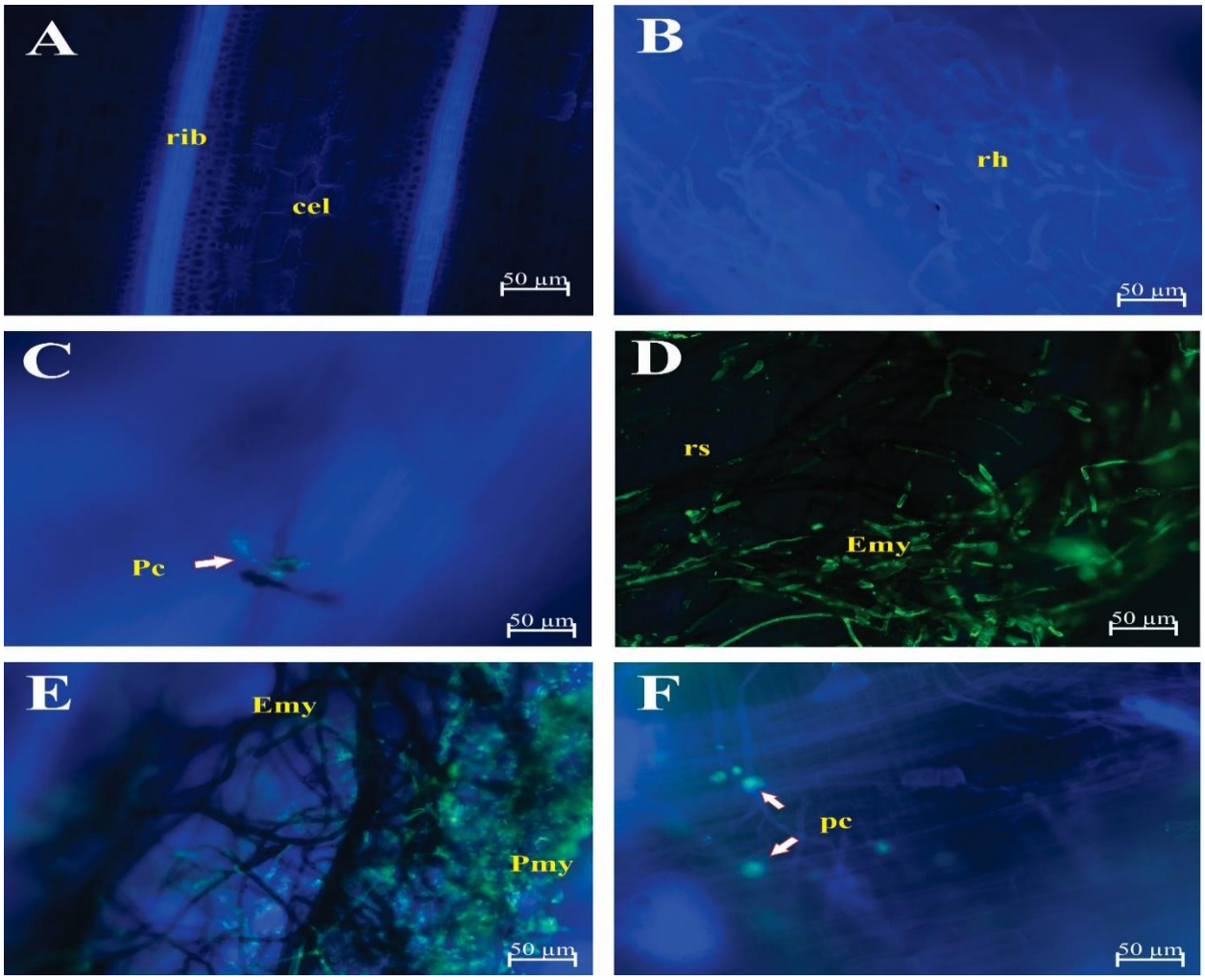

Figure 4. Epi-Fluorescence Microscope images of *Pyricularia oryzae*, *Epicoccum nigrum*, and rice plant interaction. Colonization of the vascular bundles from rice cultivars BRS-Caçula at 21 days post-inoculation (dpi) of seeds for the root, and 3 days post-inoculation (dpi) of healthy rice leaves. (A) indicate the blast negative treatment; (B) indicate the blast positive treatment in seed inoculation with *P. oryzae*, where the root hairs (rh) are observable in white; (C) indicate blast positive treatment with *P. oryzae* conidia germinating on the leaf surface (D), *Epicoccum* positive treatment (seeds inoculate with *E. nigrum*) with bright green and black indicate *Epicoccum* sp. mycelial with Alexa Flour on rice root. In (E) the bright green represents the *Pyricularia* mycelial (Pmy), and the black filamentous represents *E. nigrum* mycelium (Emy) dyed with Alexa Flour where (F) indicate the rice leaves spread with pathogen 18 dpi in the seed coated

with *E. nigrum* treatment with *P. oryzae* conidium on the leaf surface. The size bar in A, B, C, D, and E is 50 µm.

3.5. Scanning Electron microscopy (SEM) analysis

SEM analysis made it possible to observe the colony of mycelium or conidium of *E. nigrum* on the surface of the root when the rice seeds were inoculated with the suspension of BCA or both (Fig. 5D and E) (Table 1), confirming the endophytic potential of *E. nigrum.* For the positive treatment with *P. oryzae* on seeds (Fig. 5C), conidium or mycelium were not visible on the rice roots surface. Whereas, the conidium ingeminate was observed on the leaf surface for the preventive control treatment (T6) as shown on this Scanning Electron Micrograph (Fig. 5F) (see Fig. 6).

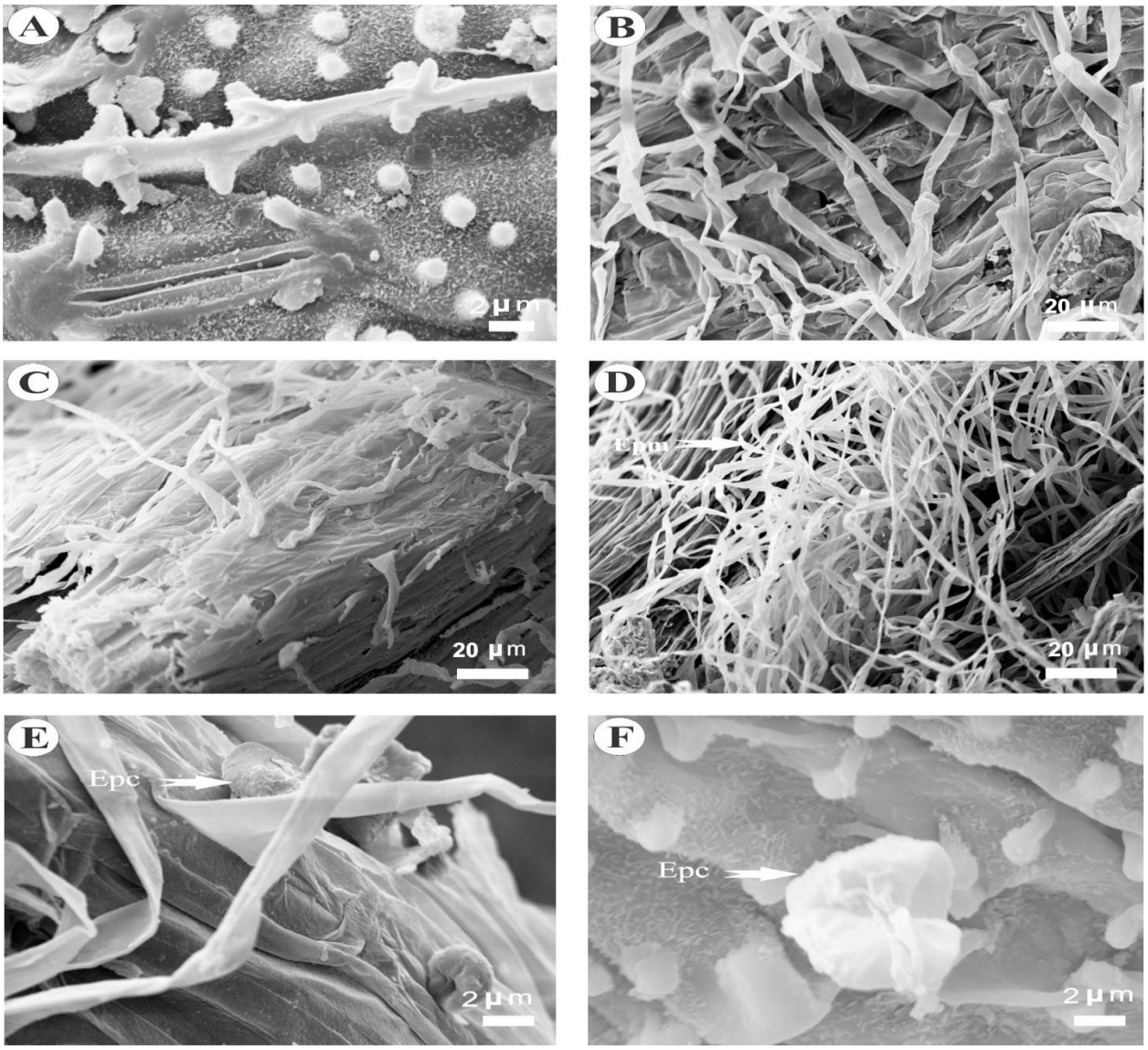

Figure 5. Scanning Electron Micrographs of *Pyricularia oryzae*, bioagent *Epicoccum nigrum*, and rice plant interaction. Colonization of the seedling of rice cultivars BRSMG-Caçula at 21 days post-inoculation (dpi) of seeds for the root, and 3 days post-inoculation (dpi) of healthy rice leaves. (A, B) indicate the negative control treatment rice leaves surface and root respectively; (C) represents the root surface of positive control treatment with *P. oryzae*; (D) indicates the positive control treatment with *E. nigrum*, and the root colonization by the BCA is observed (white arrow). (E) indicates the mixture of *P. oryzae* and BCA treatment, and *Epicoccum* conidium non-geminate is observable on the root surface. And (F), the white ball indicates *Epicoccum* conidia ingeminate on the leaf surface for the preventive control treatment. The size bar in A, B, C, D, E, and F, are 2, 20, 20, 20, 2, and 2 µm respectively, while (Epm) is *Epicoccum* mycelium, (Epc) is *Epicoccum* conidia.

3.6. Phylogenetic analysis of DNA sequence within *Epicoccum* species

The combined ITS, LSU, RPB2 and β-TUB datasets were analyzed using the Maximum Parsimony method. Sequences from each primer combination were used to obtain consensus sequences with MEGA v. 6.0. The result of the phylogeny analysis showed that the single locus phylogenies of the regions ITS, RPB2, and β-TUB placed both *Epicoccum* strains from grapevine and corn with high resolution into *E. nigrum* clade with respectively 90%, 99%, 96%. The lowest resolution was observed in the case of LSU, they were placed in the clade of *E. pimprinum* with 64% and 49% for *E. nigrum*. Analysis of the combination of the sequences of the 4 genes placed *Epicoccum* strains from grapevine and corn into *E. nigrum* clade. There were a total of 2396 positions in the final dataset. In the tree, the bootstrap support for both strain*s'* clade was 100% and the support for the clade representing *E. nigrum* was 100%.

Figure 6. Phylogenetic tree inferred from a Maximum Parsimony analysis of taxa based on analyses of a concatenated alignment of ITS, LSU, RPB2, and β-TUB sequence data of 13 strains representing the genus *Epicoccum*.

4. DISCUSSION

Epicoccum nigrum Link (syn. *E. purpurascens* Ehrenb. ex Schlecht) is an ascomycete fungus distributed worldwide, which colonizes different types of soils and host plants (Mims and Richardson, 2005). However, previous studies have identified *E. nigrum* as an important aeroallergen fungus (Bisht et al., 2002). According to Bisht et al. (2004), saprophytic fungi *E. purpurascens* found routinely in indoor and outdoor environments has been shown to cause mold allergy in 5–7% of different populations worldwide (Mims and Richardson, 2005). This may be because of the presence of cross-reactive proteins in *E. nigrum* with other species of fungi such as *Aspergillus fumigatus*, *Alternaria alternata*, *Cladosporium herbarum*, *Curvularia lunata*, *Fusarium solani*, *Rhizopus nigricans* (Bisht et al., 2002). *E. nigrum* is mainly associated with the primary decomposition of plant tissues and has been described as a weak plant pathogen of *Cucumis melo* (Braga et al., 2018). However, according to Arenal et al. (1999), *E. nigrum* is known as a highly variable species believed to represent only intraspecific variation. The polyphasic analysis established two genotypes showing morphological, physiological, and genetic divergence as well as genetic incompatibility characterized by colony inhibition, strongly indicating that the genotypes correspond to different species (Favaro et al., 2011). Despite this aspect, the genus *Epicoccum* are mainly known for their use as biocontrol agents (Braga et al., 2018). Many studies have focused on the ability of this fungus to produce antimicrobial compounds such as epicorazins A–B, epicoccins A–D (Elkhateeb and Daba, 2019), epicoccarines A–B (Bell and Karuso, 2003), and epipyridone, flavipin (Madrigal et al., 1991), and epirodins (Favaro ´ et al., 2011; Diana et al., 2012). *Epicoccum* species are famous for their application in the biocontrol of several phytopathogens their capability of producing various biologically active compounds with medical applications as antioxidant, antimicrobial, and anticancer agents (Elkhateeb and Daba, 2019), and *E. nigrum* had been used to control *Pythium irregulare* (Koutb et al., 2018), *P. debaryanum and P. ultimum* (Hashem and Ali, 2010), *Alternaria solani* (Nafady et al., 2017), *Phytophthora infestans* (Li et al., 2013), *Monilinia* spp (Larena and Melgarejo, 2009).

In this study, the strains of *Epicoccum* isolated from the grape plant were identified as *Epicoccum nigrum*, a widespread saprophytic, and reside as an endophyte in barley, oats, wheat, and corn (Fatima et al., 2016). Endophytic fungi are those which live inside of apparently healthy

and asymptomatic plant hosts (Petrini, 1991; Kusari et al., 2012). Non-pathogenic microorganisms with an endophytic saprophytic lifestyle like *E. nigrum* potentially colonize necrotic tissues so that competitive substrate colonization between different populations is common, resulting in the competition for nutrients and space (Köhl et al., 2019). Some *Epicoccum* species are considered to produce important bioactive secondary metabolites and can survive in unfavorable climatic conditions (Li et al., 2013; Nisa et al., 2015). In this *E. nigrum* experiment, the result showed no significant difference between treatments in rice seeds germination rate, whatever seed is inoculating with *E. nigrum* or with *P. oryzae* compared to the blast negative treatment. However, after germination, it was observed the contamination and the death of 5.55% of seedlings in treatment inoculated with *P. oryzae* alone, and 2.77% of the mixture of BCA and pathogen treatment. We hypothesized that seedlings could probably be contaminated by the conidium or mycelium on the soil surface after germination causing their death. In this case, Manandhar et al. (1998) demonstrated that transmission of *P. oryzae* from seeds to seedlings studied under various sowing conditions showed <4% infected plants with a seed sample with 21% infection. On other hand, Sesma and Osbourn (2004) reported a new facet of the infectious cycle of *M. grisea* which can undergo programmed development typical of pathogens infecting the roots. And, the root colonization can lead to systemic invasion and the development of classic lesions on aerial parts of the plant (Sesma and Osbourn, 2004).

This study showed that *E. nigrum* has significant antagonistic activity against *P. oryzae* when tested in greenhouse conditions. It was hypothesized that the endophytic *Epicoccum* could upregulate defense proteins and increase rice plant resistance to *P. oryzae*. Some investigation suggested that Systemic Acquired Resistance (SAR) and Systemic Induced Resistance (ISR) are two main mechanisms recognized in the resistance of plants attack by pathogens (Buchanan et al., 2015). Sena et al. (2013) reported that the crude extract of *Epicoccum* sp. applied 48 h before *M. oryzae* suppressed leaf blast by 97.6%. It led to the increasing activities of peroxidase and β-1,3-glucanase when plants were treated 24 h after application of the challenger, and the increasing activities of phenylalanine ammonia-lyase (PAL) and chitinase 72 h after pathogen inoculation (Sena et al., 2013). In the further example from Li et al. (2013), the application of conidial suspension of *E. nigrum* strain XF1 on potato leaf 1 h before inoculation with *Phytophthora*

infestans is effective in the disease control with 66–89% under greenhouse conditions, as well in field conditions. In another study, the application of *E. nigrum* as a soil mixture or seed dressing significantly alleviated the hazardous effect of *Pythium debaryanum* and *Pythium ultimum* of cotton seedlings and enhanced their vigor and growth characteristics (Hashem and Ali, 2004). Greenhouse experiments conducted by Lahlali and Hijri (2010) demonstrated that *E. nigrum* significantly improves potato yield and decreases the severity index of stem disease in susceptible potatoes. In another finding, *E. nigrum* was able to produce compounds that inhibit the *in vitro* growth of *Fusarium verticillioides*, *Colletotrichum falcatum*, *Ceratocystis paradoxa,* and *Xanthomonas albilineans* (Favaro et al., 2011). An investigation by Musetti et al. (2011) showed that apple trees treated with *E. nigrum* showed a reduction in the severity of *Phytoplasma mali* symptoms on flowers about 2 to 8 times lower compared to uninfected controls. They added that the treatment of *Catharanthus roseus* with endophyte fungi *E. nigrum* showed a reduction in the severity of symptoms caused by *P. mali* especially flowers compared to the control. Pieckenstain et al. (2001) reported that the application of *E. purpurascens* conidium reduced *Sclerotinia* head rot incidence on the greenhouse condition. According to Talontsi et al. (2013) *Epicoccum* sp. CAFTBO may produce epicolactone and epicoccolides that could show potent antimicrobial activities and significantly inhibit the effects of *Pythium ultimum, Aphanomyces cochlioides,* and *Rhizoctonia solani.* An investigation of Mari et al. (2007) showed that *E. nigrum* use as fresh or formulated cells, at a concentration of 108 conidia mL-1 were effective, significantly reducing the incidence of brown rot caused by *Monilinia* spp., compared to control, both under artificial and natural infection, from 43 to 100% in pre-harvest treatment on a nectarine.

In-plant growth promotion, a significantly higher number of tillers per plant was observed in seed soaked with *E. nigrum* compared to the untreated treatment. Also, the result suggested that seed soaking with *E. nigrum* significantly improves the number of hearts as well as plant yield, compared to the untreated one. As mentioned earlier, it was observed that the seeds soaked with E. nigrum longer than those of the control treatment at the beginning became the reverse thereafter in full tillering. We hypothesized that *E. nigrum* could act as a growth regulator of BMH.MG Caçula showed a dark green color at the ripening stage, compared to untreated. An investigation by Favaro, et al. (2011) suggested that *E. nigrum* colonizes the sugar cane surface and was capable to increase

the root system biomass.

In our study, *E. nigrum* inoculated in seed significantly increased the number of heart as well as rice production compared to the untreated treatment.

The tests on culture medium in addition to microscopy studies demonstrated that the isolate of *E. nigrum* used in this study was not pathogenic for rice BRS.MG Caçula variety. Thus, *E. nigrum* successfully colonized the rice roots surface went the seeds were inoculated with the suspension of the mixture of conidium and mycelium before sowing and demonstrating its endophytic potential. Boyle et al. (2001) suggested that host tissue colonization by beneficial endophytes may be local or systemic, inter- or intracellular, and the effects on the host could vary from asymptomatic to mutualistic symbioses. This symbiosis allowed us to know that the host plant protects and nourishes the fungus which in return produces bioactive substances, such as plant growth regulators, secondary metabolites to foster the growth and competitiveness of the host in nature and contribute positively to their host plant (Carrol, 1988). Thus, *Epicoccum* can display an endophytic lifestyle and is typically found in the inner tissues of several plant species (Fatima et al., 2016; Arnold, 2007). Furthermore, Fageria et al. (2014) reported that *E. nigrum* is an important endophytic fungus of sugarcane associated with the biological control of plant pathogens and the production of secondary metabolites. According to Fatima et al. (2016), *Epicoccum* species are saprophytes in nature, colonize and reside as an endophyte in plants, and could infect seeds from barley, oats, wheat, and corn.

Morphological examination revealed that species in the genera *Ascochyta*, *Boeremia, Didymella*, and *Epicoccum* can be accommodated in the family Didymellaceae (Pleosporales) (Jayasiri et al., 2017). It had been reported by Chen et al. (2015) that *Phoma* was delineated as three distinct genera, and the generic circumscriptions of *Ascochyta*, *Didymella*, *Epicoccum*, and *Phoma* emended. Teleomorph states of *Phoma* 100% support. However, in the case of this study, LSU region analyses placed both strains in the clade of *E. pimprinum* with 64% and 49% for *E. nigrum.* Based on this fact, we hypothesized that LSU might not be a good region to amplify in the identification of *Epicoccum* species. have been described in the genera *Didymella*, *Leptosphaeria, Pleospora,* and *Mycosphaerella*, indicating that *Phoma* anamorphs represent a polyphyletic group (Chen et al., 2015). ITS, RPB2, β-TUB, and LSU sequence data were analyzed to investigate the

phylogenetic relationships of two strains of *Epicoccum* from grapevine (*Vitis vinifera*) and corn (*Zea mays*). These multigene phylogenetic analyses provide evidence that both strains of *Epicoccum* were related to *E. nigrum* species with 100% support. However, in the case of this study, LSU region analyses placed both strains in the clade of *E. pimprinum* with 64% and 49% for *E. nigrum.* Based on this fact, we hypothesized that LSU might not be a good region to amplify in the identification of *Epicoccum* species.

5. CONCLUSION

The results demonstrate the beneficial interaction between rice cultivar BRS.MG Caçula and *E. nigrum*. The use of the endophytic *E. nigrum* successfully controlled rice blast disease while improving plant fitness and production. Therefore, this BCA could help to reduce dependence on the use of chemical fungicides and contribute to the sustainability of rice cultivation. This work emphasizes that *E. nigrum* with its excellent antifungal and biofertilizer activities can find its application as novel bio-fungicides against various phytopathogens in this field of crop management. Since a single measure could rarely provide effective, feasible levels of disease control. The effective blast disease control could certainly be the application of Integrated Disease Management (IPM) uses the combination of resistant cultivar, cultural practices, and the coating of seeds with *E. nigrum*. This result needs to be confirmed in the field.

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CHAPITRE III- Contribution of endophytic *Penicillium citrinum* in the biocontrol of rice blast disease in the greenhouse

ABSTRACT

Pyricularia oryzae Cav. is the major pathogen of rice causing blast disease significantly threatens global food security with 10% and 30% losses of the annual rice harvest. The application of endophytic fungi to reduce *P. oryzae* infections could provide a sustainable solution to reduce the amount of rice lost to blast disease and synthetic pesticides. The present investigation is an attempt to evaluate the role of endophytic *P. citrinum* trains GP1 and GP3 on the physiology, and yield of rice cultivar BRSMG Caçula infested by *P. oryzae.* The surface-sterilized rice seeds were inoculated with *P. citrinum* conidia and mycelium suspension as well as a foliar treatment. At 18 days after sowing (seedling stage of the plant), the endophytes and pathogen were inoculated on the foliage to stimulate the colonization of plant leaves depending on the treatments. *P. citrinum* strains GP1 and GP3 consortium seeds soaking or sprayed on foliage significantly decreased blast disease severity with the rates of 33.34% to 37.4% respectively. In-plant growth promotion, the consortium significantly enhanced the rice number of tillers with 11.25 against 10.17. The individual or synergetic use did not affect plant height compared to the untreated. Likewise, GP1 and GP3 individual inoculation on the seeds did not affect rice yield. Fungal isolates GP1 and GP3 both colonized rice roots tissues were identified as *P. citrinum* through phylogenetic analysis RPB2, β-TUB, and LSU regions of rDNA sequence homology. The formulation of biopesticide involving endophytic fungi comprises an environment-friendly approach to managing blast disease.

1. INTRODUCTION

Rice (*Oryza sativa* L) is by far the most important staple food for more than half of humanity, providing approximately 19% of the daily calories consumed worldwide (FERNANDEZ; ORTH, 2018). Rice is cultivated on all the continents except Antarctica, and its cultivation occupies about 23% of the total area under cereal production worldwide (FAGERIA et al. 2014). Rice production

is the main source of income and employment for more than 200 million households across the world (ASIBI et al. 2019).

Besides this fact, every plant species is frequently affected by hundreds of different kinds of fungi, bacteria, mollicutes, viruses, insects, and nematodes (LIU; WANG, 2016; AGRIOS, 2004, p. 922). For instance, more than 70 diseases caused by fungi, bacteria, viruses, and nematodes have been listed on rice (ZHANG et al. 2009). Among them, blast disease caused by fungi *Pyricularia oryzae* Cavara 1892 (syn. *Magnaporthe oryzae*) is devastating on several gramineous crops and is highly damaging to rice worldwide (NISHIMURA et al. 2016). *P. oryzae*, can infect the aboveground tissues of rice plants at any growth stage and cause total crop failure (ASIBI et al. 2019). The importance of rice blast disease is measured with different parameters such as presence in all rice-growing areas of the world, and the wide range of host plants (BALLINI et al. 2008). Despite outstanding efforts understanding the rice defense mechanisms to manage *P. oryzae*, given its great economic importance, this pathogen continues to be a major threat to global food security (LIU; WANG, 2016).

Unfortunately, neither traditional breeding nor chemical approaches have been able to contain this disease, because the pathogens can mutate to evolve resistance to multiple rice genotypes (PENNISI, 2010 p. 804-805). This fungus has caused the breakdown of several resistance conferred by newly developed commercial cultivars (CHUMA et al. 2011). The long-term approaches of alternative disease control besides pesticides are urgently required to enhance crop protection. Notably, the use of chemical fungicides and fertilizers negatively impacted the richness of the soil, its biodiversity, and therefore contribute to environmental pollution (HAJ-AMOR et al. 2020). Therefore, the biological control agents (BCAs) are applied to control plant pathogens, where they act via a range of modes of action (KÖHL et al. 2019). Most of them exhibit several mechanisms that may affect the disease triangle directly, indirectly, or synergistically (VAN DER LELIE et al. 2009), while others could improve plant growth and development directly or indirectly (ALORI et al. 2017; VAN DER LELIE et al. 2009;).

Species of *Penicillium* are ubiquitous fungi because of their undemanding nutritional requirements and their ability to grow over a wide range of conditions and environments (Tsang et al. 2018). These species have been isolated as endophytes on various plant species (TOGHUEO;

BOYOM, 2020). *Penicillium* is also known to enhance plant growth and yield by providing available phosphorus and other plant-growth hormones such as IAA (indole 3- acetic acid) and gibberellins (ALTAF et al. 2017). *Penicillium* has been reported to colonize their ecological niches and protect their host plant against multiple stresses by exhibiting diverse biological functions that can be exploited in agriculture, and biotechnology (TOGHUEO; BOYOM, 2020). Endophytic fungi are generally regarded as fungal microorganisms colonizing the internal tissues of healthy plants without causing any apparent negative effects (DING et al. 2019). These endophytes may play many important beneficial roles in the metabolism and physiology of the host plant, including induction systemic resistance to the pathogen, antagonisms activities with pathogens (TOGHUEO; BOYOM, 2020), solubilizing phosphates (LÚCIA; ENGUITA, 2016), synthesizing plant-growth hormones (Hamayun et al. 2010). Plant growth-promoting endophytes (PGPE) inhabit plant tissues and the close linkage of them inside of tissues facilitates nutrient exchange and enzymes activity (HASSAN, 2017, p. 687-695). Fourteen polyketides and six alkaloids have been isolated in the fungus *P. citrinum* (LAI et al. 2013).

Microscopy (SEM, EFM) is a helpful tool to study microorganisms (fungi, oomycete, and bacteria), their interaction with other organisms or with plants (ALVES et al. 2013). It allows studying of several aspects of the morphology, such as surface details, fungi parasitism, and saprophytism (ALVES et al. 2013). Nowadays, Scanning Electron Microscope and Epi-Fluorescent Microscope (SEM) are utilized not only in materials, chemical, and physic sciences but also in diverse fields such as medical sciences, and biology (KASHI et al. 2014). Their high spatial resolution makes them versatile and powerful equipment for the examination and analysis of a wide range of the microstructural characteristics of specimens at the nanometer to micrometer length scale (KASHI et al. 2014). It gives the three-dimensional aspect to the images, a large magnitude of increase from 10 to 1000000 times (ALVES et al. 2013).

The goal of this study is to provide details of the beneficial role of *P. citrinum* in rice blast disease suppression, its action in the improvement of plant fitness and yield while analyzing its interaction with rice plants using microscopy in pot assay under greenhouse.

2. MATERIAL AND METHODS

2.1. Plant material and microorganisms

Rice cultivar BRSMG Caçula which is considered very susceptible to *P. oryzae* in edaphoclimatic conditions in Minas Gerais state, Brazil, was used in this study. The mixture of *P. oryzae* strain A10659 and IA25 (BOTELHO, 2019 unpublished data) from respectively the collection of the Laboratory of Electron Microscopy and Ultrastructure Analysis, Department of Phytopathology (DFP), and the Department of Plant Science of UFLA, Lavras, Brazil was used in this study. The *Penicillium* isolates GP1 and GP3 assigned to *Penicillium citrinum* obtained from the Molecular Phytopathology Laboratory of the Phytopathology Department (DFP) were used in the experiments.

2.2. Molecular identification of endophytes

Molecular identification was carried out based on fungal molecular markers such as RPB2, β-TUB, and LSU of rDNA regions amplification and sequence analyses. Genomic DNA was extracted to the mycelial and conidia from the Petri dishes containing PDA and OA medium according to the method of CTAB (Cetyltrimethylammonium bromide) (LESLIE; SUMMERELL, 2006). Samples were transferred in the Eppendorf and added 750 µl of CTAB. Added 7.5 µl of β-mercaptoethanol and vortex for 2 min. Then it was transferred in the water bath at 65°C before vortex for 2 min. This process was repeated at the same temperature. 700 µl of chlorophyll (chloroform + alcohol + isopropyl 24: l) to the supernatant and vortex for 1 min before centrifugate at 13000 rpm for 10 min. Thereafter, 500 µl of the supernatant was transferred in the new tube before adding 500 µl of isopropanol as well as 50 µl of diacetate of sodium before centrifugation. 500 µl of ethanol 70% was added to the pellet and centrifugate before eliminating ethanol from the pellet in the Eppendorf. Thereafter, 50 µl of buffer TE was added to the pellet and the concentration and the quality of DNA were measured using nanodrop Lite before being kept in the refrigerator at -20°C (more details on the protocol in Appendix C). Molecular marker viz RPB2, TUB, and LSU were amplified, and sequencing was conducted by the ACTGene Molecular Analyzer DNA

sequencing services (Brazilian company), using the PCR primers for the four genes.

In phylogenetic analyses, the appropriate taxa for the analyses were initially selected following BLAST searches of GenBank (http://www.ncbi.nlm.nih.gov/). The sequence datasets were compared with similar sequences from strains of other species in the *Penicillium* species already available in GenBank and mentioned by (HOUBRAKEN et al. 2010; HOUBRAKEN et al. 2020; SAMSON et al. 2011;). An overview of strains used in this study is presented in Table 1 that were obtained from public databases. The phylogenetic analyses were done for each data set separately, as well as with a combined alignment consisting of the three RPB2, β-TUB, and LSU regions. Using the Molecular Evolutionary Genetics Analysis (MEGA) software approach we built automatically the phylogenetic tree and the evolutionary history was inferred using the Maximum Parsimony method (KUMAR et al. 2018).

Table 1- *Penicillium* spp. used for phylogenetic analysis and their accession numbers.

Species	**Culture accession number**	**GenBank accession number**	
		RPB2	**TUB**
Penicillium citrinum	CBS139.45	JN606604.1	GU944545.1
Penicillium corylophilum	CBS 330.79	JN606591.1	GU944519.1
Penicillium gorlenkoanum	CBS 408.69	JN606601.1	GU944520.1
Penicillium steckii	CBS 260.55	JN606602.1	GU944522.1
Penicillium heteringtonii	CBS_122392	JN606606.1_	GU944538.1
Penicillium tropicum	CBS 112584	JN606607.1	GU944532.1
Penicillium sizovae	CBS 413.69	JN606603.1	GU944535.1
Penicillium tropicoides	CBS_122410	JN606608.1	GU944531.1
***Penicillium* sp. GP1**	-	-	-
***Penicillium* sp. GP3**	-	-	-

2.3. Microorganisms mass production, plant growth conditions, and inoculation

Two bioagents of *P. citrinum* isolates of GP1 and GP3, individually and the consortium formed a mix of GP1-GP3 as well as two *P. oryzae* strains A10659 and IA25 were selected. Both microorganisms *Penicillium* and *Pyricularia* were grown in potato dextrose agar (PDA) and Oat-

agar (OA) medium for conidia mass production at 25 ± 2°C for 7 and 15 days respectively. Rice seeds of BRS.MG Caçula variety was prior surface disinfected with sodium hypochlorite 1% and ethanol 70% for 1min each, followed by three rinsing steps in sterile water before dried at room temperature. The inoculum source was prepared using sterilized water containing Tween 20® and the mixture *P. oryzae* strain A10659 and IA25 suspension was adjusted for the concentration of 1×10^5 conidia. mL^{-1}. Given the small and the big quantity of *Penicillium* conidia suspension, it was diluted to get the wanted concentration was 1×10^7 conidia. mL^{-1}. The substrate Trosptrato® HA Hortaliças was composed of Pinus Bark, Vermiculite, PG Mix 14.16.18, Potassium Nitrate, Simple Superphosphate and Peat. The pots of 1L of volume were filled with 800 g of the mixture of sterile sand and the rice sowing was done as shown in Table 1 and kept in a controlled greenhouse with 30°C maximum temperature. For foliar control treatments, 20 ml of *Penicillium* and pathogen inoculum had been sprayed for foliage treatment at 18 days after sowing (DAS) at the stage of one shoot with three leaves (seedling growth stage). Plants were irrigated to keep the upland conditions and optimum moisture level as per the requirement of the rice plants (FAGERIA et al. 2014). Every treatment containing six replicates was spread with 20 ml of the inoculum.

The fertilizer NPK 20-5-20 was applied at the recommended rate (90 kg. ha-1), with recommended nitrogen rate (40 kg. ha-1). Rice seedlings were incubated in a growth chamber before being sprayed with *Penicillium* and *Pyricularia,* maintained at the relative humidity of 100%, 25°C in temperature, and kept in the darkness for 24 hours to stimulate the infection. Seedlings were subsequently transferred to the greenhouse.

The experiment was designed in complete randomized design (CRD) in five treatments and seven replications, while each pot contained three plants. Two (2) replicates (pots) were used for microscopy studies, and 5 replicates for disease severity and plant growth promotion assessments.

Table 2- Treatments in *Penicillium* experiments.

Treatments	Inoculation time	Plant growth stage
T1- Negative control	Strayed water at 18 DAS	Beginning of tillering
T2- Positive control, only pathogen	*P. oryzae* 18 DAS	Beginning of tillering
T3- *Penicillium* control (GP1, GP3), seed inoculation	*Penicillium* on seed	Seed before sowing
T4- *Penicillium* (GP1, GP3) and pathogen later	*Penicillium* first, and *P. oryzae* 24 hours later 18 DAS	Beginning of tillering
T5- *Penicillium* control (GP1, GP3) seed inoculation and pathogen later	*Penicillium* on the seed before sowing and *P. oryzae* 18 DAS	Seed before sowing

NB: The experiments were performed with *Penicillium citrinum* isolates GP1 and GP3 individually and the consortium.

2.4. Statistical analysis

There was a total of six treatments, each containing 7 replicates with a completely randomized design. All data were collected and organized in spreadsheets in Microsoft Excel. All data were tested for normality and homogeneity before analysis of variance (ANOVA). Statistical analysis of variance (ANOVA) was performed using R software. The mean values were compared and separated based on the Scott Knott test served to determine statistically significant differences, which were accepted on the 95% significance level ($P < 0.05$).

3. RESULTS

3.1. Phylogenetic analysis

The regions RPB2, β-TUB, and LSU of rDNA were sequenced and analyzed. The tree obtained from the maximum parsimony analysis is shown in Fig.4.5. The evolutionary history was inferred using the Maximum Likelihood method and Kimura 2-parameter model. This analysis involved 8 nucleotide sequences. There were a total of 859 positions in the final dataset, and the evolutionary analyses were conducted in MEGA X (KUMAR et al. 2018). LSU region analysis which is rarely

used for *Penicillium* identification placed *Penicillium* isolates GP1 and GP3 in the clade of *P. citrinum* with 99% of similarity as a monophyletic group. Combination analysis was only possible with rpb2 and β-TUB regions of the sequences which placed GP1 and GP3 from grapevine in the clade *P. citrinum* with 100% of similarity. In the tree, the bootstrap support for both strains' clade was 100% (Fig.1).

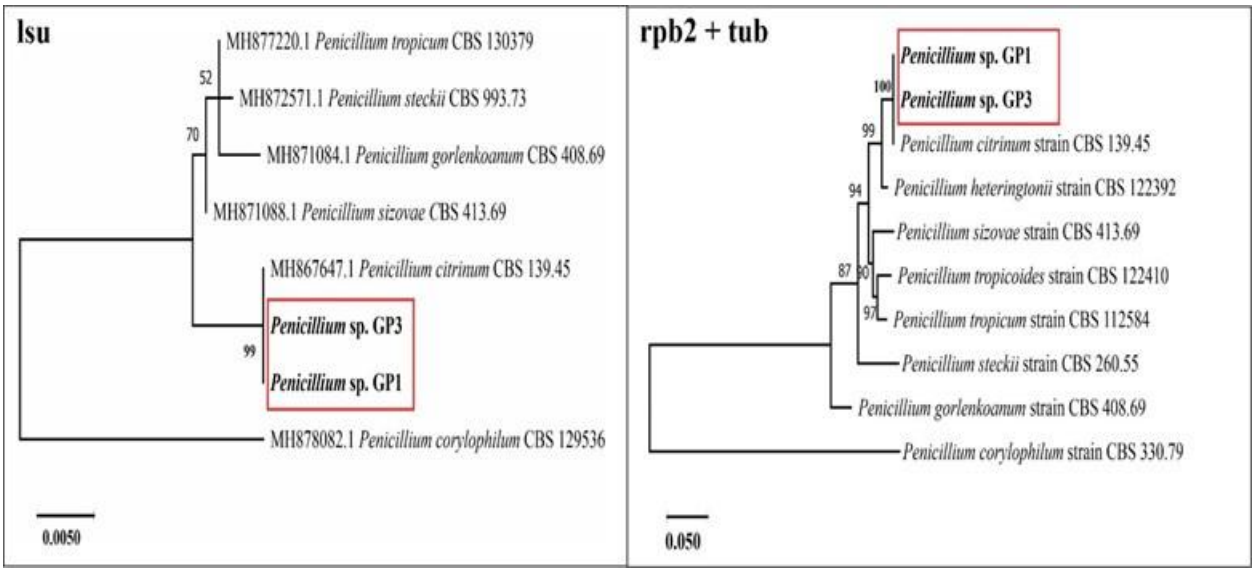


Figure 1-. Phylogenetic tree inferred from a Maximum Likelihood method of taxa based on analyses of a concatenated alignment of RPB2, β-TUB, and LSU sequences data of 8 strains representing the genus *Penicillium*

3.2. Effect of *Penicillium citrinum* in blast disease control

The result showed no significant difference between treatments in disease incidence with the index of 44.45% at the last assessment at 39 DAI (Fig. 2A). Whereas, seeds soaking (T5) with the consortium of GP1 and GP3, and its preventive foliar (T4) treatments were significantly different to pathogen positive treatment (T2) in blast severity suppression by 33.34% to 37.4% respectively (Fig. 2B). The disease incidence and severity data integrated into the area under the disease progress curve (AUDPC) over time showed no significant difference between the index. The indexes were 884.7, 875, and 865 in incidence and 324.1, 223.2, and 189.9 in severity respectively

for blast positive, seeds soaking, and foliar preventive (Fig. 2C).

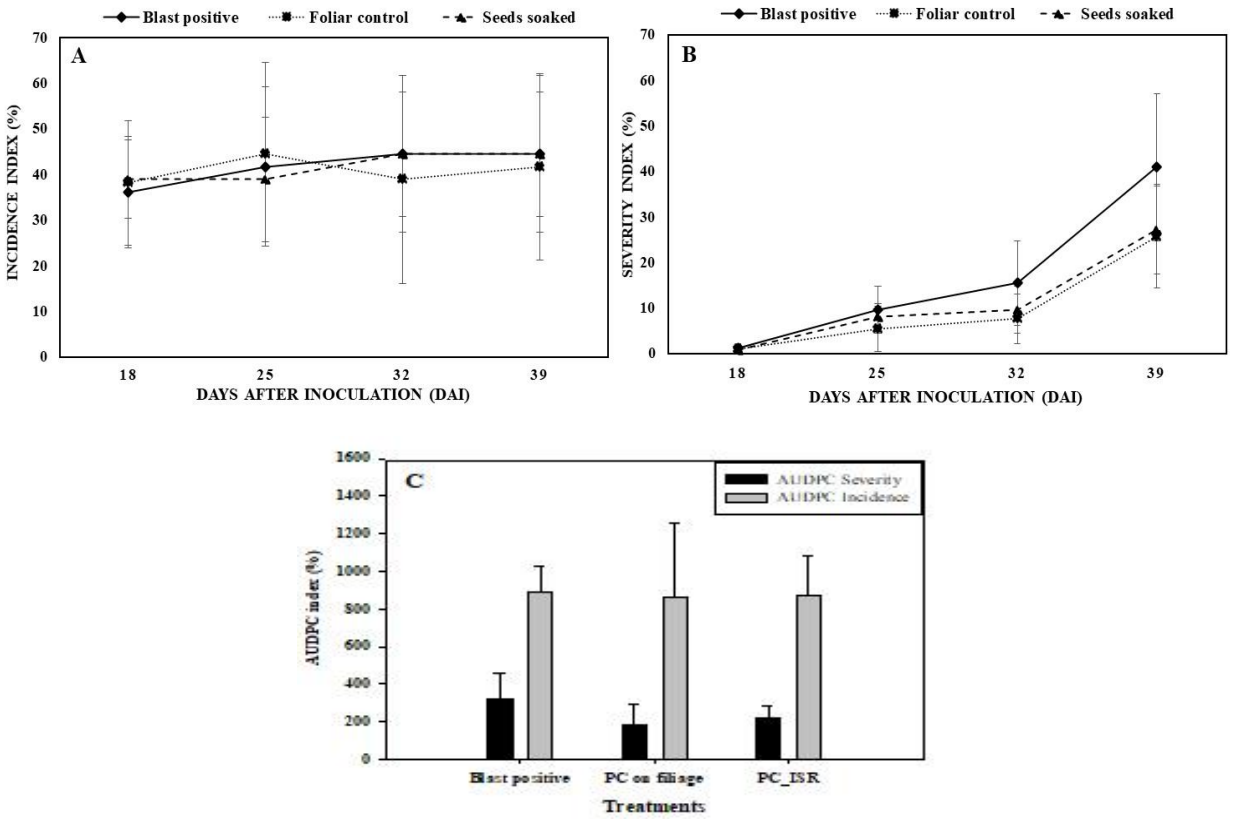
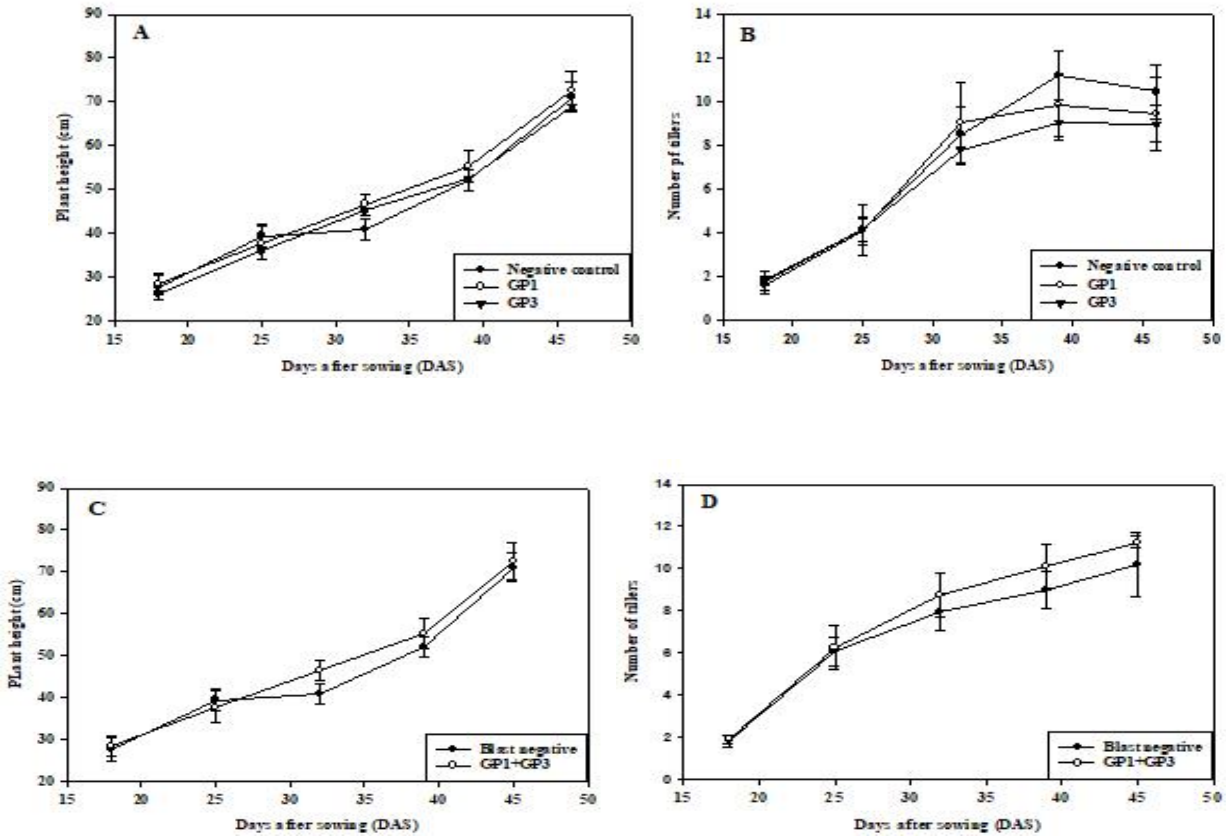


Figure 2- Effect of *Penicillium citrinum* consortium on the blast disease suppression. Figure A represents rice blast disease incidence in the function of days after sowing (DAS), and B indicates disease severity in DAS in the experiments of *P. citrinum* strains GP1 and GP3 consortium. C indicates the area under the disease progress index in incidence and severity. Bars represent the standard error of the mean.

3.3. Growth promoting and productivity activities of *Penicillium citrinum*

To investigate the effect of endophytes inoculation on plant growth, two representative endophytic *P. citrinum* isolates GP1 and GP3, plus its consortiums of GP1and GP3 were used for rice cultivar BRS.MG Caçula growth promotion. The results of ANOVA analysis indicated no significant difference between GP1, Gp3, and untreated (T1) with 72.52 cm, 72.10 cm, and 71.14 cm respectively at the last assessment at 46 days after sowing (DAS) in rice plant height (Fig. 3A).

Concerning the number of tillers, the was no significant difference between treatment with 10.48 and 9.88 and 9.08 respectively for the negative control, GP1, and GP3. Moreover, the synergistic effects of GP1and GP3 led to no significant (P-value = 0.525) difference of plant height with 70.21 cm against 67.38 cm for no inoculated plants. This result suggested that both isolates of *P. citrinum* used individually or the consortium had no influence on plants height. A significant (P-value = 0.000) enhancement of several tillers was observed (11.25) comparable with not inoculated plants (10.17) (Fig. 3D). Concerning grain production, there were no significant differences in the number of plant panicles (P-value = 0.952) with 9.61, 9.89, and 9.61 respectively for negative control GP1 and GP3 treatments. Likewise, there was no significant difference in grain weight 11.98 g, 11.65g, and 11.17g negative control, GP1, and GP3 treatments respectively (Fig. 3E). Remarkably, plants inoculated with fungal endophytes individually did not affect the number of plant height, number of tillers and panicles, as well as the productivity.



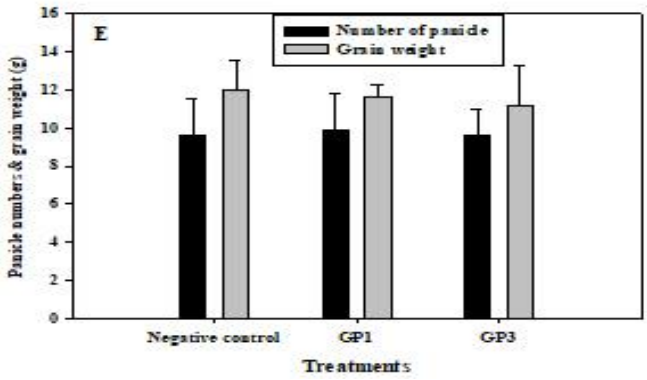


Figure 3- Influence of *Penicillium citrinum* in plant growth promotion and grain weight. (A) represent rice plant height in centimeters in the function of days after sowing (DAS), (B) indicates the number of tillers in the function of days after sowing (DAS) in both *P. citrinum* (GP1 and GP3) individual inoculation on the seed experiment. (C) represents plants height in centimeters in the function of days after sowing (DAS), and (D) is the number of tillers in DAS in the experiments of *P. citrinum* strains GP1 and GP3 consortium. (E) indicate the panicle number and grain weight. Bars represent the standard error of the mean.

3.4. Scanning Electron microscopy analysis in microorganisms-plant interaction

This scanning Electron Micrograph showed the conidia of *P. oryzae* germinating on the rice leaf surface in blast positive (T2), and ungerminated conidia in *Penicillium* preventive treatment on the leaves surfaces (T4) (Fig. 4BD). In T4 *Penicillium* conidia were observed on leaves surfaces (Fig. 4EF). Whereas, SEM analysis did not allow the observation of the colonization of roots tissues by *P. citrinum*. (Fig.4C).

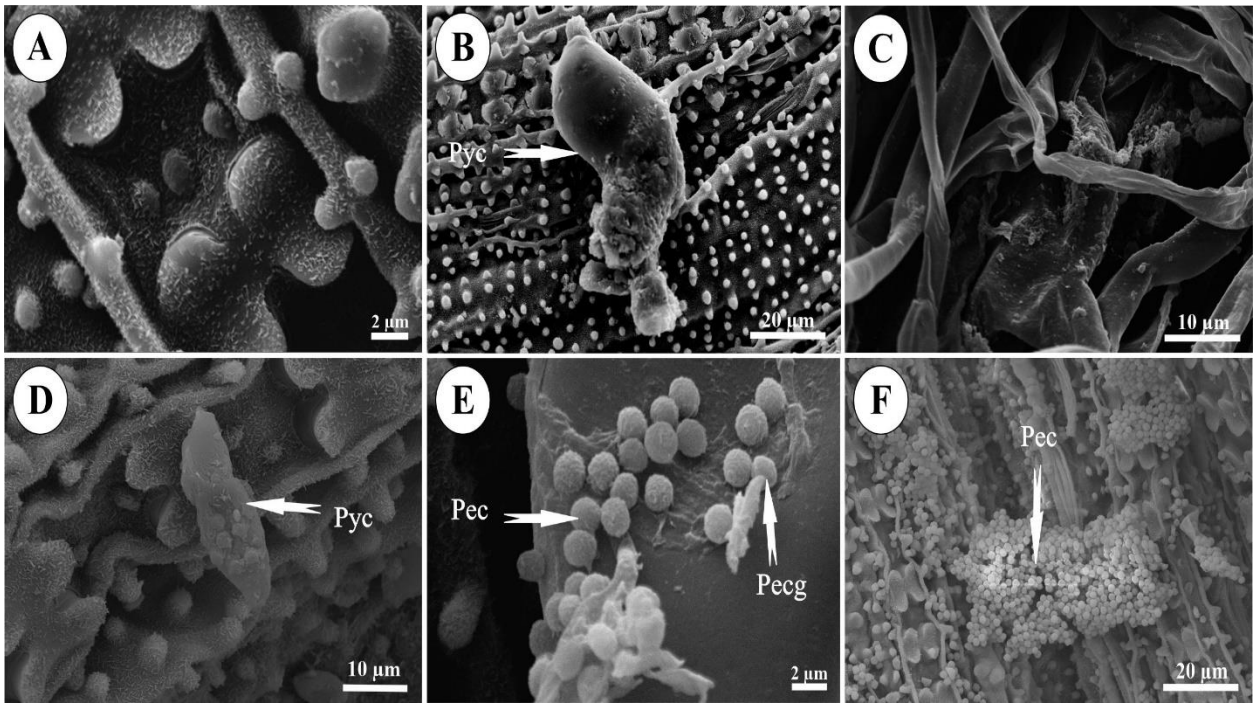


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Figure 4- Scanning Electron Micrographs of *Pyricularia oryzae*, bioagent *Penicillium citrinum*, and rice plant interaction. Inoculation of seedling of rice cultivars BRSMG-Caçula at 21 days post-inoculation (dpi) of seeds for the root, and 3 days post-inoculation (dpi) of healthy rice leaves. (A) indicates the blast negative treatment; (B) indicate *Pyricularia* conidia germinating on the rice leaf surface; (C) represents the root surface in rice seeds inoculated with *Penicillium* treatment; (D) indicates *P. citrinum* preventive inoculation on the leaf surface and *P. oryzae* inoculation at 24 hours after (T4), where *Pyricularia* conidia ungerminated was observed; (E, and F) indicates the treatment T5 with the conidium geminating and ungerminated of *P. citrinum* were observable on the leaf surfaces. The size bar in A, B, C, D, and E are 2, 20, 10, 10, 2, 20 µm respectively.

3.5. Epi-Fluorescence Microscopy (EFM) analysis

EPM analysis sowed the conidia of *Pyricularia* germinating on the leaf surface in the blast positive treatment (T2) (Fig. 5B). It allowed to observe the plant roots tissues colonization by *P. citrinum* in its seeds inoculated treatment (T3, T6) in (Fig. 5CEF). This analysis permits also to observe of *Penicillium* mycelium on the leaf surface with its inoculation as a prior treatment before challenging the pathogen (T5) (Fig. 5D). Remarkably, the EFM analysis showed strong evidence that these *P. citrinum* isolates are endophytic fungi that colonized BRSMG-Caçula roots tissues.

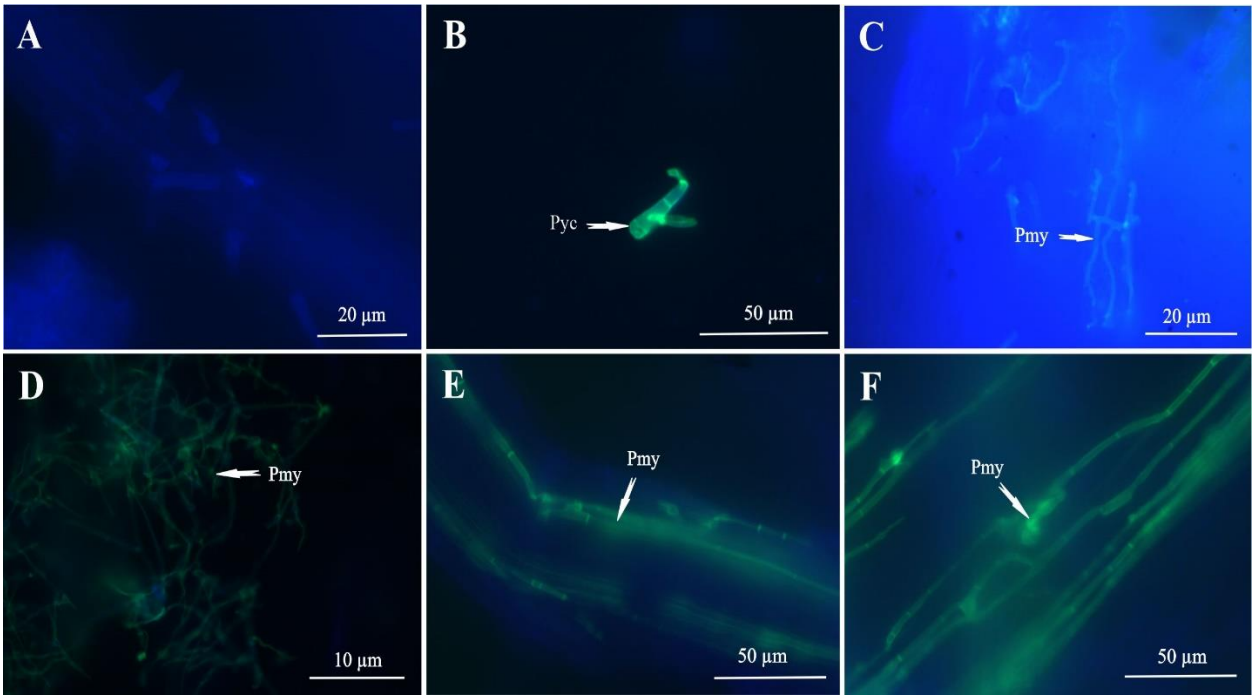

Figure 5- Epi-Fluorescence Microscopy images of *Pyricularia oryzae*, *Penicillium citrinum*, and rice plant interaction. Rice cultivar BRSMG Caçula seedling at 21 days post-inoculation (dpi) of seeds for the root, and 3 days post-inoculation (dpi) of healthy leaves. (A) indicates the root of the blast negative treatment, where the root hairs are observable in white; (B) represent the blast positive treatment in foliar inoculation with *P. oryzae*, where *Pyricularia* conidia germinating is observed on the leaf surface; (C) indicate *Penicillium* positive treatment on the rice seeds, where *P. citrinum* mycelium is observable on the root surface; (D) *Penicillium* preventive foliar treatment with bright green and indicated *Penicillium* mycelial with Alexa Flour dye. In E, and F the bright green septate mycelium represents *P. citrinum* mycelial (Pmy) dyed with Alexa Flour. The blue color of the images indicates rice leaves or roots surfaces dyed with Calcofluor. The size bars in A, B, C, D, and E are 20, 50, 20, 10, 50, 50 µm.

4. DISCUSSION

Chaverri et al. (2015) reported that the misidentification of a cryptic taxon by the use of a collective name may have far-reaching negative consequences for strategic matters in the industry, plant quarantine, and other fields, such as human and animal health. This is why RPB2, β-TUB, and LSU regions of rDNA and the Maximum Likelihood method in MEGA X (KUMAR et al. 2018) was used to identify both strains GP1 and GP3 of *Penicillium* used in this study. The analysis showed that both *Penicillium* isolates are *Penicillium citrinum* with 100% of identity.

The use of endophytic biological control agents as an alternative to chemical fungicides has attracted researchers due to their environmental safety and low cost. In the current study, the antagonistic fungus strains capable to control rice blast disease were evaluated for their antibiotic potential against a wide range of phytopathogens. The ability of *P. citrinum* to act as bioagents against *P. oryzae* was confirmed as a result of this study with 33.34% to 37.4% for seeds soaking and preventive foliar treatments respectively. It indicates the biocontrol ability of the consortium of *P. citrinum* strains GP1 and GP3 against *P. oryzae* and is in agreement with several other studies. *P. citrinum* is known for producing mycotoxin, citrinin, and cellulose digesting enzymes like cellulase and endoglucanase, as well as xylulase against pathogens (KHAN et al. 2008). According to Waqas et al. (2015), the association of endophytic *P. citrinum* LWL4 with sunflower (*Helianthus annuus* L.) decreased disease severity caused by *Sclerotium rolfsii* as compared to their respective controls. An investigation by Santhanam et al. (2015) revealed that a consortium of five bacteria was essential for the reduction of sudden wilt disease on *Nicotiana attenuata*. But, not the individual members of these bacteria associated with the roots provide lasting resistance against fungal diseases (SANTHANAM et al. 2015). Wheat yield was improved by 9% upon inoculation with a consortium of *Bacillus thuringiensis* and *Serratia* sp. (SHAKEEL et al. 2015). It was unveiled that microbe in small consortia promoted plant growth and enhance the defense signaling cascades leading to enhanced transcriptional activation of several metabolic pathways during pathogen ingress. However, an additive or synergistic effect is not achieved every time a microbial consortium is used (SARMA et al. 2015).

The negative results were obtained in plant growth parameters when GP1 and GP3 were inoculated individually. While its ability in plant growth promotion is mentioned by numerous researches (KHAN et al. 2008; WAQAS et al. 2015). The consortium of both isolates enhanced BRS.MG Caçula number of tillers with 11.25 comparable with not inoculated plants 10.17. We hypothesized that the increase in plant tiller parameter has been attributed to the synergetic effect of both strains of *P. citrinum*. In addition, the low potential of *P. citrinum* (GP1, GP3) to improve rice plant growth could reside by the fact that the dose of phosphorus was low compared to the other elements used in the formulation of NPK (20-5-20 see in the method), as this element is reported by Altaf et al. (2017) use by *P. citrinum*. It has been reported that an effective biocontrol

agent should exhibit maximum plant growth-promoting traits to benefit the crops (RAIS et al. 2016). Our results corroborate with many similar findings as it is widely reported that *Penicillium* species produce many secondary metabolites. The importance and role of soil fungi in PGP and soil health are well known (ALTAF et al. 2017). They produce a large number of secondary metabolites such as IAA, siderophores, ammonia, organic acids, antibiotics, extracellular enzymes, etc., and enhance plant growth, crop productivity, and soil fertility (ALTAF et al. 2017). Gibberellins (GAs) which is produced by *P. citrinum*, are ubiquitous plant hormones that elicit various metabolic functions required for the growth of a plant such as seed germination, stem elongation, sex expression, flowering, formation of fruits and senescence (LATIF et al. 2013). Also, *P. vinaceum* X17 has been reported by Zheng et al. (2012) as a producer of the bioactive secondary metabolites quinazoline alkaloid, which exhibited potential cytotoxic and antifungal activities. Hassan (2017) reported that endophytic fungi *Penicillium chrysogenum* and *Penicillium crustosum* were able to produce indole acetic acid (IAA) and ammonia, exhibited the capacity for phosphate solubilization, in addition to their enzymatic and antimicrobial activities.

Plant colonization by endophytic *Penicillium* is known to enhance plant growth and protect host plants against pathogens by activating plant defense mechanisms. In the present research, we showed that seed soaking with the consortium of *P. citrinum* isolates GP1 and GP3 reduced the severity of blast disease on susceptible rice cultivar BRSMG Caçula under greenhouse conditions. *P. citrinum* has been reported as a common endophytic fungus of cereal plants such as wheat and soybean (KHAN et al. 2008). As already noted, in general, endophytic colonization may improve the ecological adaptability of the host plant by enhancing resistance to biotic and abiotic stresses (AFZAL et al. 2019; YAN et al. 2019). In the present study, both isolates GP1 and GP3 of *P. citrinum* studied can be considered endophytes since they penetrated and survived in rice roots without causing observable damage. According to Boyle et al. (2001), endophytic fungi colonization of the host plant can either be systemic or localized and may have varied influences on the fungal-host interaction and could vary depending on the organ that is being colonized. Some of them are host-specific, others colonize only certain organs of the plant host (BOYLE et al. 2001). In Both partners, endophytic fungi and plants benefit from the relationship including improving the nutrient status of the host plants, influencing mineral nutrition, water absorption, growth and

disease resistance, whereas in exchange, the host plant is necessary for fungal growth and reproduction (BONFANTE; GENRE, 2010). It has been proposed that bio-compound strigolactone A, B, and C are produced by *P. citrinum* where A could whereas B inhibit plant growth (KURAMATA et al. 2007).

5. CONCLUSION

This study demonstrated that the inoculation of the consortium of endophytic fungi improved plant health by suppressing blast disease, and its fitness, therefore the nutrient uptake. The beneficial endophytic fungi could be present continuously in the host plant, which is more economic and ecological friendly plant roots gatekeepers. The formulation of biopesticide involving endophytic fungi comprises an environment-friendly approach, which could be used in combination with other approaches in IPM strategy to manage blast disease. Since *P. citrinum* is reported to supply phosphorus to host plants, further research on an in-depth understanding by increasing the phosphorus dose to allow this endophyte to express its potential more in addition to the evaluation of its efficiency in the field a. Despite this encouraging evidence, of the microbial endophyte and plant interactions could facilitate the successful application of microbe-based products.

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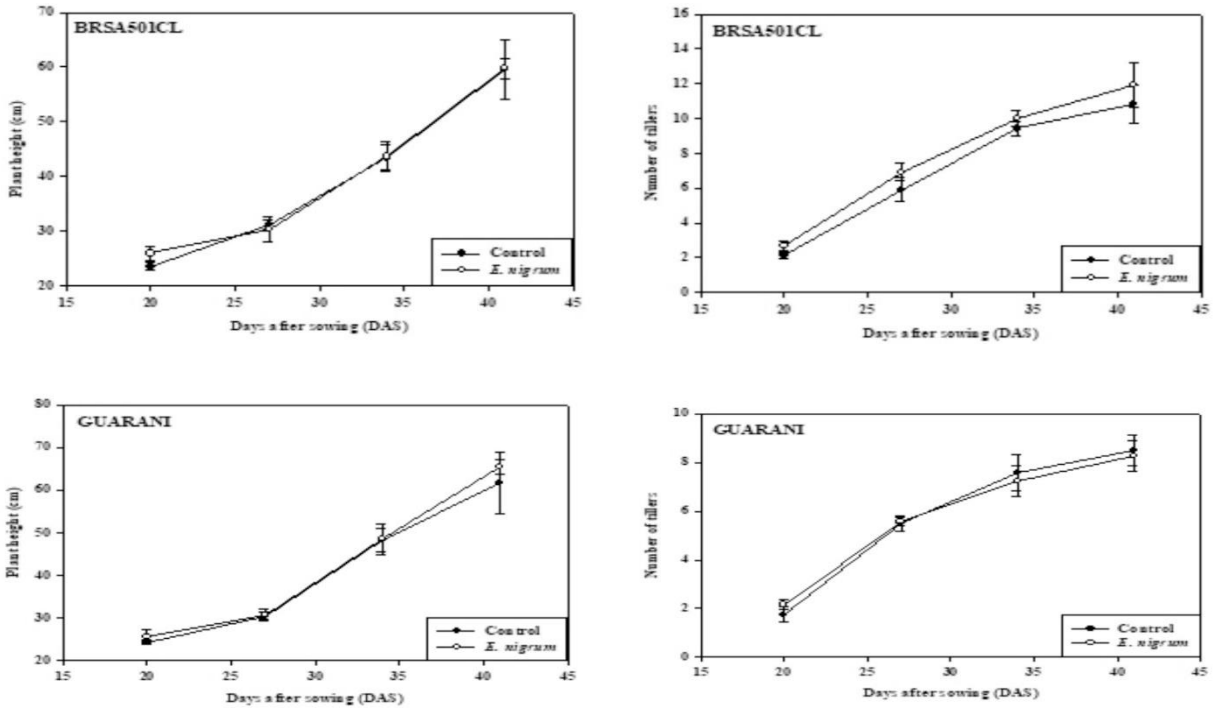
PART III- GENERAL CONCLUSION

The results obtained in this study showed the beneficial interaction between rice plants and *B. amyloliquefaciens* isolate BMH, *E. nigrum*, and *P. citrinum* in rice blast management. These microorganisms were effective in the control rice blast disease while improving plant fitness. The beneficial effects of these studied endophytes, with their antifungal and biofertilizer abilities, could find their application as novel bio-fungicides in crop management, which is a more economic and ecologically friendly approach in the context of climate change in the West African Sahel region. It must be emphasized that despite the contribution of these endophytes in the control of disease, BCAs are not a cure-all to control all phytosanitary problems. A single measure can rarely provide effective levels of disease control. Effective blast disease control could certainly get the way in the application of Integrated Pest Management (IPM) with the use of indigenous antagonistic strains associated with rice resistant cultivar, cultural practices, and reasonable fungicides application in an environment-friendly approach. In this region of the Africa Sahel with small farmers and poor, seed coating would be one of the best procedures for crop management with endophytic BCAs and their solid granule formulation would be a good way viable conservation. It is better to confirm the effectiveness of these biological control agents in natural conditions with several environmental challenges.

APENDIX

Appendix A- Test of *Epicoccum nigrum* in growth promotion of several rice genotypes

To investigate if *E. nigrum* can enhance several genotypes of rice, three genotypes viz Yinlu, BRSA501CL, and Guarani were used for this growth promotion test. In-plant height this study showed no significant difference between *Epicoccum* inoculated plants compared to the untreated with Yinlu Epicoccum treated 74.25 cm compared to 69.79 cm; BRSA501CL Epicoccum treated 59.72 cm against 59.5 cm. On the contrary, plant height in Guarani showed 65.46 cm in *Epicoccum* treated plants compared to 61.67 cm in untreated ones. Whereas in several tillers *Epicoccum* inoculated plants were significantly different 9.0, compared to the untreated with 8.25 with Yinlu cultivar. In the BRSA501CL genotype, the number of tillers of *Epicoccum* inoculated was 11.92 against 10.83 untreated. Conversely, Guarani showed 8.25 compared to 8.5 for an untreated number of tillers using the Tukey significant difference test (P-value = 0.000). We hypothesized that *E. nigrum* mays have genotype selective incompatibility.



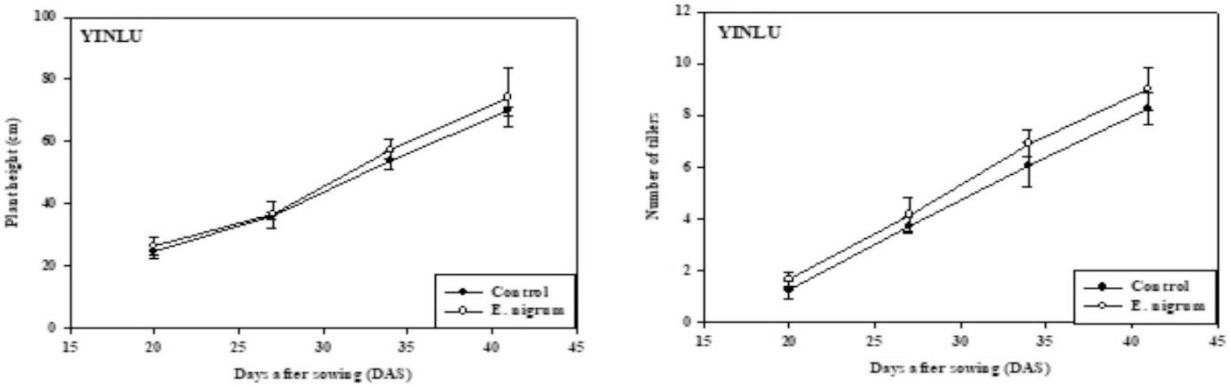


Figure 1A. Ability *Epicoccum nigrum* to enhance growth to several rice genotypes.

Rice genotypes Yinlu, BRSA501CL, and Guarani were used for the growth promotion test.

Appendix-B Influence of Epicoccum nigrum on rice plants.

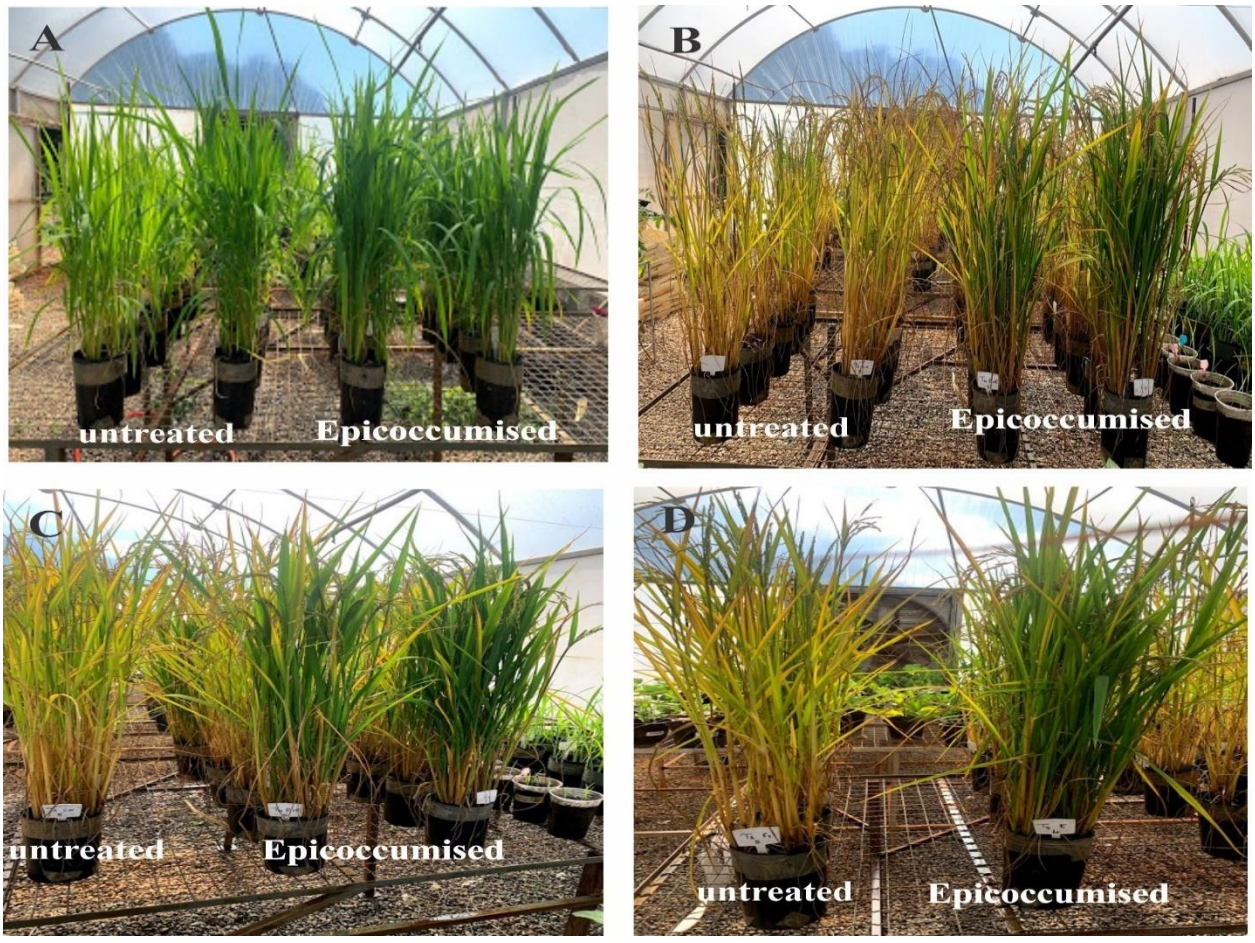


Figure 1B. Images of rice plants from Epicoccum experiment with the cultivar BRS.MG Caçula with plants 15 days before harvesting. The yellowed or light green plants indicate treatments without Epicoccum (untreated plants) (T1, T2, T6 and T7). Dark green plants represent the treatments inoculated with the suspension conidium and mycelium of *E. nigrum* (Epicoccumised) (T3 and T5) or the mixture of the suspension of E. nigrum and P. oryzae (T4). The leaves of *Epicoccum i*noculated plants conserved the green color until harvest.

Appendix C- DNA extraction protocol and polymerase chain reaction (PCR)

1- Remove the mycelium and conidium from the Petri dishes and smoke them with liquid nitrogen;
2- Put in Eppendorf and added 750 µl of CTAB;
3- Added 7.5 µl of ß- mercaptoethanol and vortex for 2 min;
4- Then put in the ban marry for 10mn at 65°C and vortex again for 2 min;
5- Put again in the ban marry for 20 nm at the same temperature;
6- Added to the suspension 700 µl of chlorophyll (chloroform + alcohol + isopropyl 24: l);
7- Vortex for 1 min;
8- Centrifugate 13000 rpm for 10 min:
9- Remove 500 µl of the suspension for another Eppendorf;
10- Added 500 µl of isopropanol;
11- Also, added 50 µl of diacetate of sodium;
12- Centrifugate again;
13- Remove the suspension from the pellet in the Eppendorf;
14- Added 500 µl of ethanol 70%;
15- Centrifugate it 5mn;
16- Discard the suspension from the pellet;
17- Dry the pellet for 20mn and add 50 µl of buffer TE before keeping in the refrigerator at 4°C;
18- Measure the concentration of the pellet with nanodrop Lite before pouring into the well of the agarose gel and keep in the refrigerator at – 20°C

PCR with markers for fungi identification

Epicoccum strain 3= 1711 ng/µl = 1.5 DNA + 48.5 H2O (water MiliQ)

Epicoccum strain 4 = 1271 ng/µl = 2.0 DNA + 48.0 H2O (water MiliQ)

Penicillium strain GP1 441.5

Penicillium strain GP3 2401.0

Example C1*V1 = C2*V2 = 1711*V1 = 50 * 50 = 1.46 µl of DNA,

Then check the concentration of the DNA with Nanodrop that must be between 100 to 20 ng/µl Applied biosystem process.

Table 1C. PCR reaction

PCR reaction	**LSU**	**RPB2**	**Tub2**	**ITS5**	**ITS4**
Gotag (Buffer)= 2.5 µl	15	20	20	20	20
H_2O =17.2 µl	103.2	137.6	137.6	137.6	137.6
MgCl2 = 2.5 µl	15	20	20	20	20
DUTP = 0.5 µl	3	4	4	4	4
Primer 1= 0.5 µl	3	4	4	4	4
Primer 2= 0.5 µl	3	4	4	4	4
Tag-Polymerase = 0.3 µl	1.8	2.4	2.4	2.4	2.4
DNA = 1 µl	1	1	1	1	1

PCR condition

95°C for 2 min
95°C for 40 s
54 °C for 30 s
72°C for 1 min
72°C for 5 min
30 time (cycle)

Preparation of the gel agarose and migration

PCR 1% = 1g of Agarose = 100 ml; X = 30 ml = 0.3 g for 30 ml of TBE

TBE Composition buffer

Tris base (0.89µ) 108g

Bore acid (0.89 µ) 55g

EDTA (0.025 µ) 9.3g

PH 8.3

Agitate (stiller) for 30 min and complete the amount of 1 land H2O MiliQ;

DNA ladder (Marker);

Buffer (DNA binding)

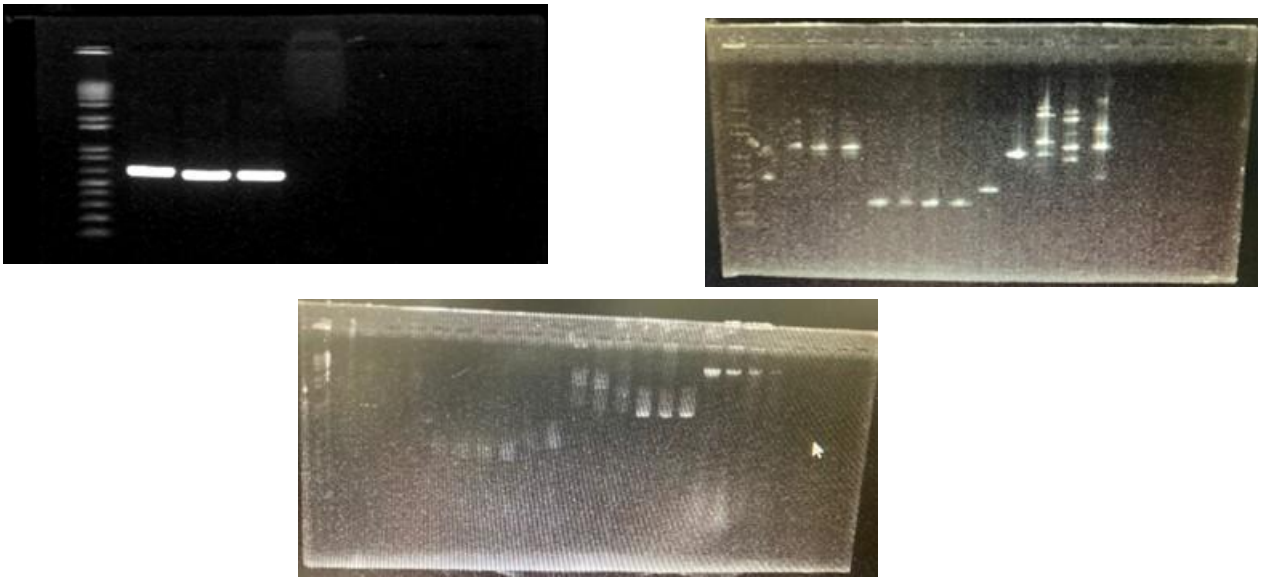


Figure 1C. Gel electrophoreses image in DNA extraction and purification of *Epicoccum* and *Penicillium*

DNA Purification by Centrifugation according to Wizard® SV Gel and PCR Clean-Up System

Gel Slice and PCR Product Preparation

A. Dissolving the Gel Slice:

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube.

2. Add 10µl Membrane Binding Solution per 10mg of gel slice. Vortex and incubate at 50–65°C until the gel slice is completely dissolved.

B. Processing PCR Amplifications:

1. Add an equal volume of Membrane Binding Solution to the PCR amplification.

Binding of DNA:

1. Insert SV Minicolumn into Collection Tube.

2. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.

3. Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

Washing:

4. Add 700µl Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

5. Repeat Step 4 with 500µl Membrane Wash Solution. Centrifuge at 16,000 × g for 5 minutes.

6. Empty the Collection Tube and recentrifuged the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution:

7. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.

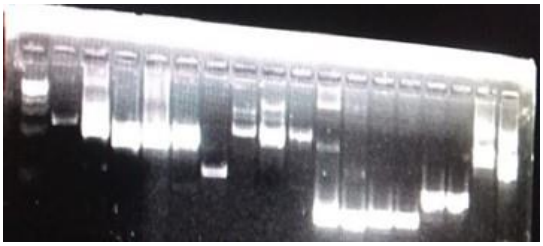
8. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × g for 1 minute.

9. Discard Minicolumn and store DNA at 4°C or –20°C

Appendix D- Protocol of RNA EXTRACTION

1- Collect leaves and keep in the refrigerator at -80%;
2- Scramble the leaves in liquid nitrogen;
3- Added 1000µl of TRIZOL in the tube with 0.1ml of scramble leaves, vortex and centrifuge 4°C/13200 rpm for 5 min;
4- Transfer 500 µl of the supernatant in the new tubes of 1,5 ml containing 200 µl of chloroform before vortex for 10 seconds and wait for 10 min;
5- Centrifuge 15mn/4°C/13200 rpm and transfer 150 to 200 µl of the upper colorless phase in the news tubes with 200 µl of chloroform again. Then vortex for second sound and wait for 10 min and centrifuge for 15mn/4°C/13200 rpm;
6- Transfer 150 µl the upper colorless phase in the news tubes of 1.5 ml containing 500 µl of isopropanol before centrifuge10 min/4°C/13200 rpm;
7- Discard the supernatant before washing the pellet with 1000µl of ethanol 75%;
8- Discard the ethanol and centrifuge 10 min/4°C/13200 rpm
9- Repeat this last process and discard again the supernatant and dry the pellet under laminar flow hoot for 30 min;
10- Resuspend with 20 µl nuclease-free water (NFW);
11- Measure the concentration of the pellet on nanodrop Lite before pouring it into the well of agarose gel.

Composition for treatment with DNase with 5 µl as the concentration of RNA for reaction and the total volume with be 25 µl, where RNA + H_2O equal 22 µl; 2.5 µl of buffer + 0.5 µl of DNase.
Incubate à 37°C for 30 min NB:
Addition 0.1 volume 2.5 µl of DNase initial reagent and mix again (It is important to homogenize the solution before use);
Incubate for 5 min at room temperature and mix all the time and centrifugate for 1.5mn/4°C/10000 rpm;
Transfer 15 µl of the supernatant in the new tubes (If the material will not be used at the same time, keep it in the refrigerator at -80°C)



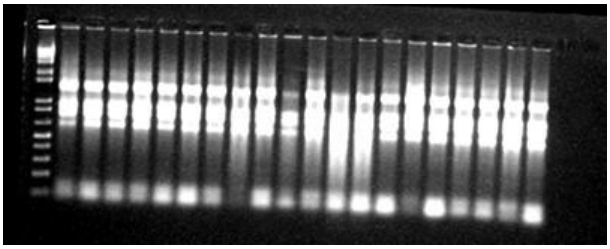


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Figure 1D. Gel electrophoreses images in RNA extraction for RT-PCR in gene expression.