RHIZOBACTERIA FOR COTTON SEED TREATMENT: SCREENING, FIELD EFFICACY AND MOLECULAR MODES OF ACTION

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Tese apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para a obtenção do título de "Doutor".

Orientador

Prof. Ricardo Magela de Souza

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MINAS GERAIS - BRASIL

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SUMMARY

GENERAL ABSTRACT

MEDEIROS, Flávio Henrique Vasconcelos de. **Rhizobacteria for cotton seed treatment:** screening, field efficacy and molecular modes of action. 2009. 101p. Thesis (Doctor in Phytopathology) – Federal University of Lavras, Lavras, MG.*

Rhizobacteria may act on eradication of seed-associated pathogens and plant protection against biotic and abiotic stresses. The present work aimed to select rhizobacteria to control cotton diseases and assess the molecular modes of action involved. A total of 368 rhizobacteria were tested for the controlo f damping-off and bacterial blight by treating infected seeds, respectively with *Colletotrichum gossypii* var. *cephalosporioides* and *Xanthomonas axonopodis* pv. *malvacearum*. The strains *Bacillus subtilis* UFLA285 and *Paenibacillus lentimorbus* MEN2 when tested for damping-off control assured germination 51% higher than the inoculated control and also controlled bacterial blight by up to 76%. In the field, strains when combined increased germination in two consecutive seasons, a result similar or higher to the fungicide control. UFLA285 also controlled damping-off caused by *Rhizoctonia solani* AG4 and significatively induced the expression of the ethylene receptor protein and peroxidase, in root and stem. Through microarray analysis, 246 genes had changed regulation, among which those related to the jasmonate/ethylene pathway, phenylpropanoids and osmorregulation. In regard to osmorregulation, proline content and aquaporin gene expression were assessed. A proline buildup was observed in infected tissues and this was higher in treated plants. The gene coding for aquaporin was down-regulated in rhizobacteria-treated and infected tissues. The rhizobacteria treatment also assured the more rapid recovery of plants submitted to a water stress and then re-watered, results obtained from photosynthesis and shoot dry weight measurements. Finally, rhizobacteria controlled diseases in cotton and the molecular mechanisms involved could be explained by the regulation of genes involved in the protection against biotic and abiotic stresses.

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*Guidance Committee: Ricardo Magela de Souza – UFLA (Advisor), Alan W. V. Pomella (Member), Paul W. Paré – Texas Tech University (Member) and José da Cruz Machado (Member).

Key words: ISR, drought tolerance, RT-PCR, PGPR

RESUMO GERAL

MEDEIROS, Flávio Henrique Vasconcelos de. **Rizobactérias para o tratamento de sementes de algodão:** seleção, eficiência em campo e modos moleculares de ação. 2009. 101p. Tese (Doutorado em Fitopatologia) – Universidade Federal de Lavras, Lavras, MG.*

Rizobactérias agem na erradicação de patógenos associados às sementes e proteção de plantas frente a estresse biótico ou abiótico. O presente trabalho selecionou rizobactérias para o controle de doenças do algodoeiro e avaliou os mecanismos de ação envolvidos. Foram testadas 368 rizobactérias para o controle do tombamento e mancha angular pelo tratamento de sementes infectadas com *Colletotrichum gossypii* var. *cephalosporioides* (Cgc) e *Xanthomonas axonopodis* pv. *malvacearum* (Xam), respectivamente. Os isolados *Bacillus subtilis* UFLA285 e *Paenibacillus lentimorbus* MEN2 garantiram a germinação de sementes inoculadas com Cgc 51% superior à testemunha e controlaram a mancha angular em até 76%. No campo, os isolados combinados aumentaram a germinação em duas safras consecutivas. UFLA285 também controlou o tombamento causado por *Rhizoctonia solani* AG4 e induziu significativamente a expressão da proteína receptora de etileno e peroxidase, tanto em raízes quanto em caules. Pelo estudo de genes expressos por microarranjo, foram observados 246 genes com regulação mudada pelo tratamento com a rizobactéria, incluindo respostas de defesa. Foram também obtidos genes associados à osmoregulação. As respostas tipicamente associadas à osmorregulação foram estudadas. Foi observado acúmulo de prolina em tecidos infectados e este acúmulo foi maior em plantas tratadas com a rizobactéria. O gene que confere para a aquaporina foi suprimido em plantas infectadas. O tratamento proporcionou o mais rápido restabelecimento de plantas irrigadas após terem sido submetidas a estresse hídrico, resultados estes inferidos pela medição da atividade fotossintética e peso seco da parte aérea. Finalmente, as rizobactérias controlaram doenças transmitidas por sementes e iniciais do algodoeiro e os mecanismos moleculares envolvidos puderam ser explicados pela regulação de genes envolvidos tanto na proteção contra o estresse biótico como o abiótico.

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*Comitê Orientador: Ricardo Magela de Souza – UFLA (Orientador), Alan W.V. Pomella (Coorientador), Paul W. Paré – Texas Tech University (Coorientador) e José da Cruz Machado (Coorientador).

Palavras-chave: ISR, tolerância à seca, RT-PCR, PGPR

CHAPTER I:

General Introduction

1 Importance of cotton and the impact of diseases

Cotton (*Gossypium hirsutum* L.) is the most important fiber producing crop and in Brazil, it is responsible for the fifth most cultivated area in the world (Cotton Incorporated, 2008). However a discrepancy in yield among growing regions, even among highly productive regions such as Goiás (2631t/ha), compared to Mato Grosso (3380t/ha) suggests a need to improve the presently encountered growing strategies, notably those related to disease control (Suassuna & Coutinho, 2007).

Furthermore, the cultivated area is a result of an increase in cultivated acreage since the last 10 years and this has resulted in an increase in the agrochemical usage (Campanhola & Bettiol, 2003). The cotton cultivation presently represents 10% of agrochemical usage in Brazil (Sindag, 2006) with fungicides accounting for 31% of all agrochemicals presently applied (Suassuna & Coutinho, 2007).

Increased demand for organic cotton has been reported. Cotton consumer countries such as United States and England are expected by 2013 to impose that 10% of all purchased cotton originate from organic source (Myers & Stolton, 1999).

The requirement for a reduced level of fungicide use and an increasing demand for organic cotton imply will serve as an impetus for alternative disease control strategies can minimize fungicide application in agriculture.

2 Cotton diseases and their importance

More than 250 etiological agents of disease have already been reported in cotton (Cia & Salgado, 1997), fortunately not all result in significant damage that would be worth consideration for disease control programs. The distribution of important diseases is also dependent on the growing regions. For

instance, two of the presently important diseases in Brasil, ramulose and ramularia spot, are not found in the North American cotton fields.

Cotton plants are either grown in successive or alternate growing seasons (Hulugalle & Scott, 2008). In either case, litter from the harvest or plants that remain alive from one season to the next serve as a continuous plant-microbe contact in the soil which can lead to the selection of highly virulent strains and early disease outbreaks. In the case of ramularia mildew spot the disease has shifted from a disease that only infects plants at the end of the season to a disease of major concern that has been reported to produce symptomatic plants as early as 30 days after sowing (Utiamada et al., 2003).

Aside from ramularia, damping-off and ramulose are the most widespread diseases in Brazil, bacterial blight, although a less severe pathogen because of the use of resistant cultivars still represents a problem for cotton growers. In spite of its importance, little is known about ramularia spot, neither the pathogen nor the disease, and control strategies rely mainly on screening of effective fungicides in the field. This project focuses on damping-off, ramulose and bacterial blight which are either seed transmited and/or infect seedlings at early development.

2.1 Damping-off

Regardless of the region, cotton growers suffer from damping-off outbreaks which may necessitate the replanting up to 10% of the cultivated area (Goulart, 2005). The revenue loss exclusively due to damping-off and consequently lower yield has been estimated as 27% (Kirkpatrick & Rothrock, 2001), the disease etiology is diverse, but is more commonly caused by *Rhizoctonia solani* (Goulart, 2005). The pathogen infects cotton plants and overwinters as sclerotia and clamidospores or can exist as saprofitical growths on decaying organic matter (Manian & Manibhushanrao, 1990). Eventually plants may overcome

the infection but the pathogen can build up in the soil leading to later outbreaks under propitious conditions.

Damping-off can occur during pre or post-emergence, with pre-emergence being more commonly reported (Kirkpatrick & Rothrock, 2001). Pre-emergence is assessed in plant stands and post-emergence by fallen seedlings. Lesions are initially light brown, lengthwise and located at the root-shoot interphase. Girdling-like lesions rapidly progress inward and clock-wise, reaching the xylem and causing seedling damping-off or wilting. Eventually the pathogen grows throughout the hypocotyls leading to wire-like symptom.

Although, *Rhizoctonia solani* Kühn has been reported as the most common damping-off causing pathogen, others such as *Pythium* spp, *Fusarium* spp., *Colletotrichum gossypii* South (var. *cephalosporioides* Costa) have also been reported as causing the same disease symptoms and it is not impossible that growers would assign the observed symptom to *Rhizoctonia* without a thorough investigation of the disease etiology.

2.2 Ramulose

C. gossypii var. *cephalosporioides* is the causal agent of ramulse. Initial symptoms are observed in younger leaves, characterized by circular necrotic spots that tear necrotic tissue apart in a star-like manner. Once the leaf develops, an unbalanced growth is observed with the pathogen hampering growth on the infected side of the leaf. Immediately after the first lesions appear, the pathogen rapidly colonizes the main meristem, killing the mainstem, leads to an excessive branching and witches's broom-like symptom due to the apical dominance (Cia & Salgado, 1997). This effect works as a source of new tissue for fungal infection and drain of nutrients that would foster flowering, thus leading to both increased flower abortion and reduced size of

formed bolls (Suassuna & Coutinho, 2007). Failure of disease control causes up to 75% reduction in yield (Cassetari Neto & Machado, 2005).

2.3 Bacterial blight

One of the most important cotton diseases in the past was bacterial blight caused by *Xanthomonas axonopodis* pv. *malvacearum* (Vauterin et al., 1995). The pathogen is easily disseminated and hardly hampered once established. Bacterial blight occurs widespread throughout cotton growing regions where susceptible cultivars are employed such as Deltapine Acala 90 and DP90B (Chitarra, 2005). Although resistant cultivars are currently effective in protecting cotton against bacterial blight in Brazil, a highly virulent bacterial blight strain has challenged cotton resistance programs in Africa (Chakrabarty et al., 1997).

Symptoms on leaves are initially green water soaked lesions limited by the veins giving an angular shape. The lesions evolve a light brown color with necrotic areas merging with neighbor, overtaking the total leaf. The pathogen infects all plant parts with round-shaped lesions, oily on the edges and necrotic in the center observed on the bolls. Under high inoculum pressure, the pathogen infects petiole, peduncle and stems (Cia, 1977).

3 Importance of seed in disease transmission

From the growers to the plant pathologists, all agree that seeds provide the starting material for most cultivated crops and its intrinsic genetic make up and overall health will determine the vigor and productivity potential of the crop (Borém, 2005).

From a health perspective, the ideal seed would is free plant pathogen (Goulart, 2005). However, for most crops the pathogen damage threshold for seeds has yet to be determined and/or techniques sensitive enough to determine

seed health are not available. In addition, there are pathogens not present in a certification program, but represent a clear risk to certain crops (Dhingra, 2005).

Since low populations of a pathogen when associated with crop seeds can result in considerable losses, the sensitive detection of plant pathogens in seeds is critical. For example, *Colletotrichum lindemunthianum* in common bean does not damage the seed or the embryo, but can build up its population after germination to reach epidemiological levels early in a plant development under favorable conditions (Machado & Pozza, 2005).

In cotton, the importance of seed transmission on disease development has recently been examined in the field (Araújo, 2008). A close correlation between initial pathogen inoculum and final disease incidence was observed. The higher the inoculum pressure of *C. gossypii* var. *cephalosporioides* associated with seeds influenced the disease incidence 40 days after sowing, with the higher disease incidence observed on bolls. Although evidence has not yet pointed out that a direct relationship between infected bolls and seed infection, infected seeds are observed to have up to a 33% chance of disease transmission (Goulart, 2005).

Another cotton pathogen reported as being seed transmitted is *X. axonopodis* pv. *malvacearum.* Although less efficient in transmitting the disease, since only 4% of infected seeds result in infected plants (Cia & Salgado, 1997), bacterial pathogens can reach epidemiological threshold levels more rapidly. In cotton, the number of infected seeds that results in epidemic outbreaks has yet to be established however for *X. vesicatoria* risk assessment, one infected seed in a 10,000 seed-lot resulted in 100% bacterial spot incidence in bell pepper under favorable environmental conditions (Carmo et al., 1996), such as the one that prevails in tropical growing regions (Al-Dahmani et al., 2003).

Seeds that remain in the soil after harvest can provide the initial inoculum for the following growing season and infected seed survival has been reported to be sustained for up to three years (Cia & Salgado, 1997).

4 Alternative disease control

Considering the importance of seeds in the transmission of pathogens and the need to reduce fungicide loads in the environment, seed treatment may profice a practical and cost efficient strategy to reduce seed-born pathogens as well as pathogens that the plant would have to face in the early seedling development whether soil- or air-borne such as *Rhizoctonia solani* (Machado et al., 2000).

The seed treatments in use rely on fungicide and more than one compound is used to achieve a broad spectrum disease control. Nevertheless, replanting can be required due to damping-off outbreaks (Goulart, 2005) and an absence of effective seed treatment specifically targeted to control bacterial blight.

Therefore, the search for alternative disease control strategies, to be either combined with presently used agrochemicals or having a broad spectrum activity would improve stands, reduce epidemic disease outbreaks later in the growing season would improve overall yield.

From an alternative disease control perspective, the rhizobacterium-based strategies have proved to be effective in cotton (Brannen & Kenney, 1997). In a survey of biological products for the disease control, most of the microrganisms used in biological control are bacterium-based (51%), and the most common genus found was *Bacillus* sp (41%) (Montesinos, 2003). It is not surprising this genus is more commonly used, since it has unique properties that readily allow for commercialization. It has considerable phenotypic plasticity, growing from 15 to 60^{o}C and perhaps more important Bacillusspp. Produces

endospores for survival even beyond the mentioned temperatures or under scarce nutrient conditions (Lamanna, 1940). Interestingly, *Bacillus* spp produces a diversity of metabolites with broad spectrum activity (Schisler et al., 2004).

While surviving on the leaf surface is a challenging environment because of the exposure to UV light and sudden changes in humidity and temperature throughout the day (Dickinson, 1971), in the soil and especially in the rhizosphere, bacteria encounter a more stable niche for development and to exert disease control (Cook & Baker, 1983). As it is with fungicides, biological seed treatment is cost-effective for eradicating pathogens from seeds and protecting plants from infection (Cook & Baker, 1983).

As previously stated, seed treatments aim at eradicating pathogens from seeds and protecting germinated plants from infection. For biological-based seed treatment, a similar ability is found. Within the bacterial growth, antibiotic and resistance elicitors are produced, which lead to fewer pathogens and plant protection, respectively (Romeiro, 2007).

With maize, a *Bacillus* sp seed treatment has been effectively used to reduce fungal levels below detection limites (Luz, 2001). In field studies, seed coated plants with *Bacillus* sp were 5 and 9% more able to control fungal levels than seed treatment with the fungicides Thiram and Iprodione, respectively.

After seed germination, the bacterium survives using root exudates. In contrast to agrochemicals, which degrade in the soil, biological control agents are able to provide sustainable protection. For example, cotton seed treatment with *Bacillus cereus* controlled cotton damping-off and can be recovered up to 72 days after planting (Pleban, 1995).

Improved protection against plant pathogens by benefitial soil bacteria can also be achieved by combining two or more bacterial strains (Jetiyanon et al., 2003).

5 Modes of action of biocontrol agents

Rhizobacteria exert biological control through three main mechanisms: antibiosis, induced systemic resistance or competition (Romeiro, 2007) and each mechanism has its distinct characteristics such as time for response, dose response, nature of molecules involved, systemicity of the response and duration of the effect.

In cotton, biological control of various diseases has been examined (Mondal & Verma, 2002). Treatment with *Bacillus* spp against bacterial blight resulted in a disease control of 45% (Arya & Parashar, 2002; Ishida et al., 2008).

While Arya & Parashar (2002) found that disease control occurred when plants were treated with the antagonist two days before challenging with the pathogen, there was a dose-response and antibiosis was the mechanism involved.

On the other hand, Ishida et al. (2008) reported that at least seven days were necessary from treatment with the antagonists and the inoculation, to achieve successful control, there was no dose-response and the mechanism involved was exclusively based on the induction of defense-related responses.

Antibiosis and induced systemic resistance strategies can be present in the same biological control agent as is found with *Bacillus subtilis* M4 in the control of damping-off (Ongena et al., 2005). The combined antibiosis and induced resistance has also been reported in *Bacillus subtilis* GB03, which is commercially marketed as Kodiak (Brannen & Kenney, 1997; Ryu et al., 2004). This later bacterium has also been implicated in growth promotion (Zhang et al., 2007) and the observed growth promotion in the field for *Bacillus*-treated peanut has been related in part to the 37% average increased yield over a multi-year trial (Turner & Backman, 1991).

In *Bacillus subtilis* at least 20 antibiotic metabolites have been identified and an estimated 5% of the bacterial genome is allocated to the production of such antibiotics (Stein, 2005).

Although most of the work on the efficacy of such bacterial metabolites has focused on disease control, such compounds may also be important players in ecological adaptability, not only by assuring exclusive presence in the rhizosphere but also facilitating spread and colonization on roots and inducing systemic resistance, as reviewed by Ongena & Jacques (2005).

6 Other benefits exerted by rhizobacteria

Rhizobacteria have been reported in the control of pathogens in a wide range of plant species (Mondal & Verma, 2002) and have also recently been shown to induce abiotic stress tolerance.

Arabidopsis thaliana plants exposed to high salt tolerance (100mM NaCl) exhibited growth similar to plants cultivated in salt-free medium and this has been explained by the down-regulation of the sodium transporter (HKT1) in *Arabidopsis thaliana* (Zhang et al., 2008).

Another soil bacterium has been shown to induce drought and salt stress tolerance in the same plant (Cho et al., 2008) and the key molecule involved in this induction (2,3-butanediol) has already been shown to induce systemic resistance in *A. thaliana* against *Pectobacterium carotovorum* subsp. *carotovorum* (Ryu et al., 2004), a common bacterial soil-borne pathogen.

7 Microarray technique to assess plant-microbe relationships

In order to explain changes mediated by rhizobacterium, pathogen, or both, interacting with the plant, researchers have been using microarray.

The technique allowed Zhang et al. (2007) to probe how growth promotion is mediated by *Bacillus subtilis* strain GB03 via changes in organ specific auxin distribution in *Arabidopsis thaliana*.

In cotton, Dowd et al. (2004) explained changes after infection by *Fusarium oxysporum* f.sp. *vasinfectum,* a wilting pathogen, demonstrating the presence of disease resistance as well as drought stress tolerance gene over-expression. The cotton microarray chip has been updated based on all deposited *Gossypium* spp ESTs (Udall et al., 2007) although new has not been published on the plant pathogen interactions or tritrophic interactions between cotton, a cotton pathogen and a biological control agent.

A commonly produced *Bacillus* spp surfactin, a cyclic lipopeptide is thought to be involved in induced systemic resistance (Ongena & Jacques, 2008). Tobacco cells cultivated in a medium containing micromolar concentrations of this protein induce defense-related enzymes including phenylalanine ammonia lyase and lipoxygenases as well as modified phenolic patterns (Jourdan et al., 2009). This metabolite induction was correlated with calcium influx and dynamic changes in protein phosphorylation but not associated to phytotoxicity or adverse effect on the integrity of treated cells (Jourdan et al., 2009). Thus, these lipopeptides may interact with reversible pore formation in a way sufficient to induce disturbance or transient channeling in the plasma membrane that could in turn activate biochemical cascades of molecular events leading to defensive responses (Jourdan et al., 2009). The two other commonly produced antibiotic active molecules: iturin and fengycins did not have any activity on plant defense (Jourdan et al., 2009).

When the biocontrol agent, *Trichoderma hamatum,* was used in a formulation to treat tomato seedlings, a total of 45 foliar genes were found to have changed regulation. Those genes were mainly associated to changes in plant physiology such as biotic and abiotic stresses and, since only a

pathogenesis related protein (PR5) was found to be up-regulated, an assumed modulation of metabolism-related genes were reported as responsible for the observed control of bacterial spot (Alfano et al., 2007).

Part of the changes in metabolism may represent a shift in the primary metabolism directing the production of microbial active molecules by the plant. Cartieaux et al. (2003) observed that *Arabidopsis thaliana* plants originated from seeds treated with *Pseudomonas thivervalensis* strain MLG45 were more resistant to *Pseudomonas syringae* by the over-expression of defense-related responses such as peroxidases and chitinases, but this positive reponse was accompanied by a reduced photosynthesis and growth.

The induced defense responses have been reported as having consequences on plant growth (Heil, 2001). However, this detrimental side effect of induced resistante is not always present, since growth promotion and activation of defense related genes were observed in *Arabidopsis* plants exposed to GB03 volatile organic chemicals (Ryu et al., 2004; Zhang et al., 2007). For each particular situation, the observed gene expression should be checked by phenotype analysis.

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CHAPTER 2:

Broad spectrum disease control using *Bacillus* spp.-based cotton seed treatment

1 ABSTRACT

Biological seed treatment has a broad spectrum disease control and activity from seed to field levels. Looking for alternative disease control strategies, 368 endospore-forming bacterial strains were screened for bacterial blight and damping-off control, caused by *Xanhomonas axonopodis* pv. *malvacearum* and *Colletotrichum gossypii* var. *cephalosporioides*, respectively. Consistent disease control with seed treatment was found in two strains: *Bacillus subtilis* UFLA285 and *Paenibacillus lentimorbus* MEN2 with expressed disease symptoms reduced 45 and 56%, respectively for damping-off and 26 and 76%, respectively for bacterial blight. Bacterial populations were recovered from bacterially treated seeds (10^3cfu/g) with increased germination rates over a two-year field trial. The greatest improvement in disease control and seed germination was observed for seeds treated with both strains in combination, a result similar or higher than the recommended fungicide.

Key-words: biological control, *Gossypium hirsutum*, sinergism, PGPR

2 RESUMO

O tratamento biológico de sementes tem amplo espectro de controle de doenças e atividade tanto ao nível de semente quanto de campo. Buscando-se estratégias de controle alternativo de doenças do algodoeiro, 368 isolados bacterianos formadores de endósporo foram selecionados para o controle da mancha angular e tombamento, causados por *Xanhomonas axonopodis* pv. *malvacearum* e *Colletotrichum gossypii* var. *cephalosporioides*, respectivamente. O controle das doenças pelo tratamento de sementes foi reprodutível quando usadas duas bactérias: *Bacillus subtilis* UFLA285 e *Paenibacillus lentimorbus* MEN2 com expressiva redução nos sintomas da doença de 45 e 56%, respectivamente para o tombamento e 26 e 76%, respectivamente para a mancha angular. As populações bacterianas foram recuperadas de sementes tratadas (10^3ufc/g) com aumento nas taxas de germinação em dois anos de ensaio. O mais alto aumento na resistência à doença e germinação de sementes foi observado para sementes tratadas com a combinação de ambos isolados, um resultado igual ou superior ao tratamento com o fungicida recomendado.

Palavras chave: controle biológico, *Gossypium hirsutum*, sinergismo, PGPR

3 INTRODUCTION

An increase in cotton (*Gossypium hirsutum* L.) cultivation in Brazil of the last 10 years to currently being the fourth largest producer on an area basis has been accompanied by substantial increases in agrochemical applications. Ten percent of all active ingredients for Brazilian agrochemical applications is for cotton (Campanhola & Bettiol, 2003) and 31% is targeted for fungal disease control (Sindag, 2006), alternative disease control strategies such as biological control agents are imperative(Myers & Stolon, 1999).

In cotton, biological control against foliar and soil-borne diseases has already achieved some success (Mondal & Verma, 2002). For bacterial blight, *Bacillus* sp-based plant spray has resulted in a 40% control level. While Arya & Parashar (2002) found that the disease control occurred when plants were treated with the antagonist two days before inoculation, there was a dose-response and antibiosis was the mechanism involved.

On the other hand, Ishida et al. (2008) reported that at least seven days were necessary from leaf treatment with the antagonist and the inoculation to achieve successful control, there was no dose-response and the mechanism involved was exclusively based on the induction of defense-related responses.

Antibiosis and induced systemic resistance strategies have been found associated with disease protection by *Bacillus subtilis* M4 against damping-off (Ongena et al., 2005) as well as *B. subtilis* GB03, marketed commercially as Kodiak against a variety of plant pathogens (Brannen & Kenney, 1997; Ryu et al., 2004).

The rhizobacterium *B. subtilis* GB03 has also been implicated in growth promotion (Zhang et al., 2007) and the observed growth promotion in

the field for treated peanut has been related in part, to a 37% average yield increase over a multi-year trial (Turner & Backman, 1991).

Several genera of rhizobacteria have been reported as having the potential to control plant diseases and promote plant growth but one has gained more insight because of its peculiar survival traits, *Bacillus* spp. This genus has considerable phenotypic plasticity, i.e. growth from 15ºC to 60ºC and endospore formation for survival beyond the mentioned temperatures or scarce nutrient availability (Lamana, 1940); under favorable conditions it grows rapidly and is able to tolerate anaerobic growth and produces a diversity of metabolites with broad spectrum activity (Schisler et al., 2004). Therefore, *Bacillus* strains that have been screened for disease control have more successfully passed through the commercialization process than gram negative/non-sporulating genera (Emmert & Handelsman, 1999).

Although biocontrol agents have been criticized for their specificity to a particular host plant or cultivar (Enebak et al., 1998) such as *B. cereus* originally isolated from *Sinaps* sp, that controls cotton disease and survives as a root endophyte for up to 72 days (Pleban, 1995).

UV light, temperature and humidity fluctuation associated with the leaf surface provide a less stable environment for bacterial growth than what is encountered in the rhizosphere (Dickinson, 1971). Biological seed treatment is cost-effective for eradicating pathogens from seeds and protecting plants from infection (Cook & Baker, 1983). In the biological seed treatment of corn, Luz (2001) did not recover fungi from treated seeds and field plant stands were 5 to 9% higher than fields treated with the fungicides Thiram and Iprodione, respectively.

In cotton, several pathogens represent important threats to crop yields including the seed transmitted diseases caused by *Xanthomonas axonopodis* pv. *malvacearum* (Smith) (Vauterin et al., 1995) (bacterial blight) and

Colletotrichum gossypii (South) var. *cephalosporioides* (Costa & Fraga Jr., 1937) (damping-off and ramulose) (Cassetari Neto & Machado, 2005).

For most of the cotton growing regions, resistant cotton cultivars largely control against most pathogens, however diverse and widespread pathogen races as well as the planting of susceptible cultivars such as Deltapine Acala 90 and DP90B increase the potential for disease outbreaks. Other seed-transmitted pathogens cause both damping-off and/or ramulose (Cassetari Neto & Machado, 2005) and although chemical treatment efficiently controls damping-off, foliar fungicide sprays targeting ramulose are often used throughout the plant cycle (Chitarra et al., 2008).

Presently, no rhizobacterium-based commercial product targets both bacterial and fungal pathogens in cotton. The broad spectrum activity of selected antagonists have been obtained by screening strains for both pathogen groups and/or combining selected microorganisms for a synergistic effect against multiple pathogens (Jetiyanon et al., 2003).

The present work aimed at screening endospore-forming bacterial strains for cotton seed treatment to control both bacterial blight and damping-off. Here we report the activity of selective rhizobacterium strains in field growth promotion, germination and post-emergence damping-off with a two-year field trial.

4 MATERIALS AND METHODS

4.1 Screening for *Bacillus* spp strains

In order to obtain efficient antagonists for the control of cotton disease, rhizobacterium strains from research centers or isolated from root and soil samples (Table 1).

For isolation samples were collected from plants in 200 sites among the most important cotton growing regions in Brazil, i.e. Primavera do Leste, Campo Verde, Rondonópolis, Alto Taquari (Mato Grosso State); Chapadão do Céu and Montevidiu (Goiás State); Chapadão do Sul and Costa Rica (Mato Grosso do Sul State) and Patos de Minas (Minas Gerais State). Plants were sampled either because of their higher height or healthy leaves compared to the neighbouring ones, both benefits reported as part of the plant-rhizobacterium association in the field (Pleban, 1995). Only sites where cotton had been grown for at least four years and seedlings were up to 30 day-old were considered in order to assure that the obtained rhizobacteria would have been adapted for growth using the cotton root exsudates and that the bacterium would survive for up to the time of a regular seed treatment (Huang et al., 2008).

The screening was exclusive for endospore-forming by heating the sample to 80^{o}C for 10 min, according to Bettiol (1995). Samples used in the screening were from the roots (endophytes) and rhizospheric soil (epiphytes). For roots, endophytes were isolated based on the method described Barreti et al. (2008), where the surface sterilization is performed twice and the sterility check is done by plating 0.1mL of the last wash solution.

TABLE 1 Rhizobacteria either obtained from research center or isolated from cotton roots or rhizospheric soil and used in the screening for strains with the potential to control damping-off and bacterial blight, caused respectively by *Colletotrichum gossypii* var. *cephalosporioides* and *Xanthomonas axonopodis* pv. *malvacearum*.

Strain species and code	**Original host[3]**	**Deposited**	**Detentor**
Bacillus spp. UFLA 1-208[1]	*Gossypium hirsutum*[a]	UFLA, Lavras, MG	Ricardo Souza
Bacillus spp. UFLA227-423[2]	*G. hirsutum*[b]	UFLA, Lavras, MG	Ricardo Souza
Paenibacillus lentimorbus MEN2	*Cucumis melo*[a]	UFRPE, Recife, PE	Rosa Mariano
Bacillus sp. RAB9	*Raphanus sativus*[a]	UFRPE, Recife, PE	Rosa Mariano
B. cereus L2-1	*G. hirsutum*[b]	UFLA, Lavras, MG	Ricardo Souza
B. subtilis ALB629	*Theobroma cacao*[a]	Mars Center for Cocoa Science, Itajuípe, BA	Fabio C. Chaves
Bacillus sp. SEM1	*G. hirsutum*[b]	UFLA, Lavras	Ricardo Souza
Bacillus subtilis AP3	*Oryza sativa*[b]	Embrapa CNPMA, Jaguariúna, SP	Wagner Bettiol
Bacillus subtilis AP5	*O. sativa*[b]	Embrapa CNPMA, Jaguariúna, SP	Wagner Bettiol

[1]UFLA1-208 represents a total of 208 *Bacillus* spp. strains within this range of codes and isolated from rhizospheric soil; [2]UFLA227-423 represents a total of 153 endophytes strains within this range of code and isolated as endophytes from roots; [3]For each plant host, the superscript letter stands for niche from where it was isolated, epiphyte (a) or endophyte (b)

Only one out of the most abundant and phenotypically similar colonies was considered for each sampled site. Isolated bacteria were only used in the experiments if having the desired traits, adapted from Romeiro (2007): (1) endospore-forming, by warming the bacterial suspension at 80^{o}C for 10 min and confirming growth, (2) gram staining, the expected bacteria are gram positive and the test also make it possible to check for colony purity. Bacterial strains meeting those requirements were preserved in glycerol (40%) at -80^{o}C until use.

After screening, unidentified strains were identified based on the 16S ribosomal rRNA using the primer combination 8F (5'-AGAGTTTGATCATGG-3') and 1492R (5'-TACCTTGTTACGACTT-3'), designed based on the *Escherichia coli* 16S rRNA (NCBI deposited sequence), following previously described DNA extraction and PCR protocols (Barretti et al., 2008).

4.2 Seed inoculation

In order to test the ability of biocontrol agents to control seed-borne pathogens *Xam* and *Cgc*, cotton seeds cv Deltapine Acala 90 were initially disinfested for 2 min in sodium hypochloride (2% active chloride), washed thoroughly with sterilized distilled water, dried under cabinet flow, inoculated with each of the pathogens, treated with the biocontrol agents and assessed for each tested disease.

For *Cgc*, the fungus obtained from the Seed Pathology Lab (DFP – UFLA), isolate coded Cgc1, was grown from purified colonies on PDA at 25 ºC, 12h light for eight days, after which each plate was soaked with 5mL distilled sterilized water, transferred to sterilized 10mL-test tubes, homogenized in vortex and the obtained fungal suspension was adjusted to 10^{5}conidia/mL. To grow the fungus on new plates, 200µL of the suspension was spread on 9cm

Petri dishes containing PDA amended with manitol (69.4g/L) to yield a -1MPa water potential, which had previously shown not to interfere in fungal growth and had not allowed seeds to germinate (Machado et al., 2004). After five days of fungal growth, sterilized seeds were transferred to the fungal mat surface (25 seeds per dish) for 72h, then removed from the dishes, dried in a flow cabinet for 4h and immediatly used or stored in paper bags at 4^{o}C for no longer than two months (Tanaka & Menten, 1991). For all new inoculations, the pathogen was recovered from infected seeds. A non-inoculated control was composed by incubating seeds over the same water restriction medium for the same period of time but without the pathogen matt.

For *Xam*, following the method previously described (Medeiros et al., 2007), the bacterium, strain IB1153 was isolated from herbarized previously infected leaves and individual colonies were spread-plated on 523 medium (Kado & Heskett, 1970) for 48 h and then the suspensions were prepared to yield 10^{8} ufc/mL ($0.7A_{520nm}$). The sterelized seeds were transferred to a 500mL-Beaker and added to the bacterial suspension (2mL/g seed). The Beaker was placed inside a dissicator, connected to an air pump through a hose and vaccum pressure applied at 40cm lead (Hg) for two minutes, the hose was suddenly disconnected to despressurize the system and the whole process was repeated to assure seed inoculation. Seeds were dried and stored in a way similar to that described for Cgc-inoculated seeds. A non-inoculated control was made by damping seeds in a saline buffer solution (0.85% NaCl) and subjecting them to the vacuum infiltration, the same way the inoculation was performed.

4.3 Seed treatment and planting

The screening for the best strains in the control of seed-borne cotton diseases was performed for each one of the pathogens in order to obtain

bacterial strains able to control both pathogens or to combine strains effective for each one of the pathogens.

For each assay, the preserved rhizobacteria were transferred to agar nutient-containing test tubes and after 48h growth at 25ºC, cells were harvested by scrapping the bacterial mat and used to prepare the bacterial suspension in saline buffer at 10^8cells/mL in Neubauer chamber. The bacterial suspension was used to treat seeds by immersion (2mL/g seed) for 30min, the suspension was drained out and the bacterium allowed to colonizing seeds overnight (12h) before planting. A positive control was made by treating non-inoculated and a negative control by treating infected seeds with saline buffer at the same rate used for the antagonist suspension (2mL/g seed).

Treated seeds were sown in commercial potting mix Plantmax (Eucatex, São Paulo), in disposable 500mL-pots filled at full capacity. A total of 5 seeds were sown per pot and three replicates of one pot each. For the tested pathogens, the considered variables were seed germination at 15 days after sowing and disease severity at germination and every three days up to 15 days after sowing. In the case of Cgc, severity was determined using a rating scale from 0 to 3, where (0) symptomless seedlings; (1) superficial lesion on cotyledons covering from 1 to 25% leaf area; (2) lesion representing 26 to 50% leaf area (3) lesion representing more than 50% leaf area (Teixeira et al., 1997) and for Xam was used the 0-4 scale adapted by Ishida et al. (2008), where (0) not visible symptom, (1) 1-25% infected leaf area, (2) 26-50% infected leaf area, (3) 51-75% infected leaf area and (4) more than 75% infected leaf area. Disease severity was transformed to the disease index (McKinney, 1923) for each pot and used to calculate the area under the disease progress curve (AUDPC) (Shanner & Finney, 1976). Data for germination and AUDPC per ´pot was submitted to variance analysis and comparsion of means according to the grouping test (Scott Knott) using SISVAR.

The screening for each disease was split into five batches and the best significance group of each one was tested again in a series of three experiments, each one containing all the selected as better than the negative control.

4.4 Microbe recovery after biological seed treatment

Seeds naturally harbor pathogens that may have detrimental effect on germination speed, stand and/or early plant epidemic outbreaks and the seed treatment, reduces the pathogen population below a control level (Machado, 2000).

In order to assess the diversity and percent recovery of microorganisms associated to seeds after treatment, plants were treated with each selected bacterial strain or water similar to that described in the seed treatment section. For each replicate 25 seeds were laid over 11cm-diameter Petri dishes containing agar (20mL at 15g agar/L) amended with 2.4-Dicholorophenoxiacelic to avoid germination. After seven-day incubation at 25^{o}C with 12h photoperiod, the blotter test was analyzed for the diversity inferred from morphological markers and total percent of recovered fungi per treatment. The experiment was carried out in a complete randomized design with four replicates, each represented by one dish containing 25 seeds each.

4.5 Effect of seed treatment on the disease control and growth promotion in the field

Each 100kg of seeds cv Fibermax 993 were treated with either water (0.6L), a combination of the fungicides fludioxonil at 300mL (Maxim, Syngenta) and carboxynilide+dimethylditioncarbamate at 300mL (Vitavax-thiram, Dupont), or each selected bacterial strain, isolated or in combination, at 0.6L of each bacterial suspension at 2x10^{9} endospores/mL or a combination of both strains (MEN2 and UFLA285) by mixing 0.3L of bacterial suspension at

the same concentration used for the bacteria alone. The bacterial concentration used in the field trial was higher than the one used for the screening experiments based on preliminary tests to overcome the lower volume used but maximum used by cotton growers for seed treatment in the field.

Insecticides were used to avoid damping-off caused by insects, using thiamethoxam (Cruizer 600FS, Syngenta) at 600mL/100kg of seeds.

As part of the cotton grower used seed treatment, seeds were also treated with the post-emergence herbicide protectant, diethyl-phosphothioate (Permit 500 DS, FMC) at 1.2kg. All mentioned products were mixed with either water, fungicide or each rhizobacterial strain to make a final volume of 1.2L and seeds were treated by transferring the combination of treatments to a platic bag containing a known amount of seeds and shaking vigorously until an homogenous color of seeds was observed.

Since the bacterial strains had not been tested for nematodes or white mold (*Sclerotinia sclerotiorum*) and both diseases were potential trends based on previous year epidemics, an in-furrow application at 30L/ha of a combination of the nematicide carbofuran (Furadan 350SC, FMC) 1L/ha and the *Trichoderma asperellum*-based product Quality® (Laboratório de Biocontrole Farroupilha, Patos de Minas, Brazil) at 100g WP/ha and the dispersing agent (SAG, Syngenta) at 0.015L.

Sowing was performed using an automated tractor-propelled 10-row planter where fertilizer, according to recommendation for each planting season and in-furrow treatment, was simultaneously amended. The experiment was arranged in a randomized block design with six blocks and each plot encompassed five-30m long rows, where only the core three-10m long rows were considered for germination assessment.

At 15 days after sowing, plots were assessed for germination, calculated by dividing the germinated seedlings per meter by the set sowing

density (9 seeds/m). By that time, the number of fallen seedlings, as a result of post-emergence damping-off, was also assessed and calculated as percent fallen seedlings/total germinated ones (including the fallen ones).

At 30 days after sowing, plants were assessed for growth promotion. For each replicate, 20 plants were harvested, over-dried at 70^{o}C for three days and the shoot was weighed for each plant individually and averaged for each replicate.

All evaluated variables, i.e. germination, pot-emergence damping-off and shoot dry weight were submitted to variance analysis and for significant effects, means were compared according to Tukey's test at $P \leq 0.05$, using SAS®.

5 RESULTS

From all samples collected, endospore-forming bacteria were recovered in the rhizospheric soil (10^4 to 10^6 cfu/g) and as endophytes from roots (10^2 to 10^5 cfu/g).

In the screening for damping-off control, the strains UFLA285 and MEN2 (Table 1) showed significant disease control results compared to the control, after the experiment was repeated three times (Table 2).

One of the strain species was not known and the identification was performed by homology of 16SrRNA with deposited sequences. The DNA isolated from the bacterial strain, when amplified using the primers described in the Materials and methods section yielded a 500bp-fragment, which was sequenced and after Blasting with NCBI deposited sequence. It was identified as *Bacillus subtilis* (NCBI accession number, sequence will be deposited after manuscript publication approaval) with a 97% homology to type species.

Both strains assured germination higher than inoculated control and this result (80%) was similar to the actual seed germination potential in the absence of the pathogen (non-inoculated control). The disease, measured by the area under the disease progress curve, was reduced by 59 and 45%, respectively by *Paenibacillus lentimorbus* MEN2, *Bacillus subtilis* UFLA285 and the shoot dry weight for seedlings treated with either pathogen was similar to the non-inoculated control and higher than the inoculated one.

TABLE 2 Control of damping-off caused by *Colletotrichum gossypii* var. *cephalosporioides* in cotton (*Gossypium hirsutum*), measured by the area under the disease progress curve (AUDPC), germination and shoot dry weight 15 days after sowing through seed treatment with selected rhizobacteria: *Bacillus subtilis* UFLA285, *Paenibacillus lentimorbus* MEN2, non-inoculated or treated and inoculated untreated controls.

Treatments[1]	**AUDPC**[2,5]	**Germination**[3, 5] **(%)**	**Shoot dry weight**[4, 5] **(g/plant)**
Bacillus subtilis UFLA285	250.0 b	80 a	0.17 a
Paenibacillus lentimorbus MEN2	187.5 c	80 a	0.22 a
Positive control	-	80 a	0.17 a
Negative control	458.3 a	53 b	0.06 b

[1]treatments encompassed each selected bacterial strain (UFLA285 and MEN2), a positive control represented by hypochloride desinfested non-inoculated and treated with water and a negative control represented by inoculated seeds treated with water; [2]area under the disease progress curve (AUDPC), was calculated according to Shaner & Finney (1977); [3]germination was calculated as the number of seedlings per pot with severity below 2 at the 15th day after sowing; [4]shoot dry weight of seedlings harvest 15 days after sowing and oven dried at 70^{o}C until constant weight; [5]means are average of three experiments that were satistically similar and were analysed collectively, means followed by the same letter in the column are similar according to Tukey's test ($p \leq 0.05$)

The same set of 368 endospore-forming strains was tested for bacterial blight control. While some strains had expressive control of bacterial blight (data not presented) they did not have any effect on damping-off control and the purpose of testing the rhizobacteria for the control of both strains was to have a broad spectrum disease control and both MEN2 and UFLA285 controlled not only damping-off but also bacterial blight (Table 3). As with damping-off, the

disease control was measured by the AUDPC and a reduction of 26% and 74% was observed respectively for UFLA285 and MEN2.

TABLE 3 Control of bacterial blight caused by *Xanthomonas axonopodis* pv. *malvacearum* on cotton (*Gossypium* hirsutum L.) seedlings measured by the area under the disease progress curve (AUDPC), at 15 days after sowing through seed treatment with selected rhizobacteria: *Bacillus subtilis* UFLA285, *Paenibacillus lentimorbus* MEN2, non-inoculated or treated and inoculated untreated controls

Treatments[1]	**AUDPC**[2]
Bacillus subtilis UFLA285	29.3 b
Paenibacillus lentimorbus MEN2	10.2 c
Negative control	39.6 a

[1]tratments encompassed the bacterial strains and a negative control represented by infected seeds treated with water; [2]area under the disease progress curve (AUDPC), calculated according to Shaner & Finney (1977), means followed by the same letter are similar according to Tukey's test ($p \leq 0.05$)

One of the possible mechanisms involved in the control of plant disease is antibiosis (Romeiro, 2007) which is the direct activity of the antagonist on the pathogen. Healthy non-sterilized cotton seeds were treated with the selected antagonists (MEN2 and UFLA285) isolated or in combination, and observed for the fungal and bacterial population. None of the recovered bacteria were yellowish and creamy, a peculiar feature of most *Xanthomonas* spp due to the production of xanthomonadin (Poplawski et al., 2000). After treatment with the bacteria alone or in combination, regardless of the tested antagonist, a similar bacterial population was found (10^3cfu/g), while in the water treated control a much lower population was recovered (10^1 cfu/g).

When assessing the fungal diversity (Table 4), no pathogen was identified but most of the observed ones had already been reported as involved in seed decay (*Aspergillus* spp. and *Penicillium* spp.) (McGee, 1995). The percent of recovered fungi was also different among treatments. The combination of strains (UFLA285+MEN2), resulted in the highest reduction in fungal population (76%), followed by UFLA285 (60%) and MEN2 (29%) tested alone.

Since the selected strains showed broad spectrum disease control under controlled greenhouse conditions, they were tested for the disease control in the field. The experiment was carried out in an area with history of epidemics of both damping-off and ramulose and where bacterial blight had previously been reported in Patos de Minas, Brazil. Since, no epidemiological risk could be taken by growers, cultivars susceptible to bacterial blight could not be used since the disease was potentially prevalent, hence the cv. Fibermax 993 was used instead. The experiment was conducted in two growing seasons (2008 and 2009) and the disease severity in each year was significatively different ($P<0.05$) which did not allow the combined analysis.

For both tested years, germination was significatively higher when the combination of the selected strains was used, with increases of 8% and 37% in germination compard to the water treated control for the first and second year, respectively (Table 5).

TABLE 4 Biological cotton seed treatment with either *Bacillus subtilis* UFLA285 or *Penibacillus lentimorbus* MEN2 alone or in combination reduced fungal and increases bacterial population associated to seeds

Treatment[1]	**Recovered bacteria (log_{10} cfu/g)** [2,3]	**Recovered fungi**[2,3] **(%)**	**Diversity of recovered fungi**
UFLA285+MEN 2	3,25 b	4.0 c	*Cladosporium spp., Aspergillus ochraceus*
UFLA285	3,00 b	6.8 c	*A. flavus*, *A. niger*, *Penicillium* spp.
MEN2	3,25 b	12.0 b	*A. niger*, *Cladosporium* spp.,
Control	1,00 a	17.0 a	*A. ochraceus*, *A. niger, A. flavus, Penicillium* spp., *Cladosporium* spp.

[1]Seeds were treated with each of the selected antagonists alone at 0.6L bacterial suspension ($2x10^9$cfu/mL)/100kg seeds or in combination at 0.3L of each antagonist at the same concentration as they were used alone. All treatments were used along with the cotton grower's agrochemicals: herbicide and Thiamethoxam (Cruizer 600FS, Syngenta) (600mL/100kg seeds), diethyl-phosphorothioate (Permit 500 DS, FMC) (1.2kg./100kg seeds); [2]Means are average of 25 seeds and four replicates per treatment; [3] means followed by the same letters in the column are similar according to Tukey's test ($P \leq 0.05$)

TABLE 5 Germination and post emergence damping-off as a result of tested cotton seed treatments: *Bacillus subtilis* UFLA285 (UFLA285) and *Paenibacillus lentimorbus* MEN2 (MEN2) alone or in combination, the fungicide or the water treated controls

Treatments[1]	**Germination (%)**[3,4]		**Post-emergence damping-off (%)**[3,4]		**Shoot dry weight (g)**[3,4]
	Seasons		**Seasons**		
	2008	2009	2008	2009	
UFLA285 + MEN2	82.5 a	66.1 a	1,67 a	10.74 a	1.80 bc
UFLA285	76.3 b	60.7 b	1,83 a	8.84 a	2.80 a
MEN2	66.7 c	56.3 c	2,00 a	11.28 a	1.31 c
Fungicide	75.1 b	66.4 a	3.83 b	8.29 a	2.30 ab
Water control	76.2 b	48.1 c	4,50 c	9.86 a	2.29 ab

[1]Seeds treated with each of the selected antagonists at 1.2L bacterial suspension (10^9cfu/mL)/100kg seeds, Thiamethoxam (Cruizer 600FS, Syngenta) (600mL p.c./100kg semente), diethyl-phosphorothioate (Permit 500 DS, FMC) (1.2kg./100kg seeds). 30L/ha of *Trichoderma asperellum* (Quality) at 100g/ha and carbofuran 1L/ha was apllied in-furrow; [2]Germination = percent ratio between germinated seedlings and the sowing rate (9 seeds/m) Post-emergence damping-off = percent ratio between the number of fallen seedlings and the total number of seedlings (including the fallen) both variables analyzed 15 days after sowing; [3] Average shoot dry weight of 20 plants per replicate and six replicates per treatment harvested 30 days after sowing; [4] Means followed by the same letter in the columns are similar according to Tukey's test ($P \leq 0.05$).

The local cotton grower used fungicide control (Maxim+Thiram), resulted in germination similar to the control for the first year and to the combination of rhizobacteria (MEN2+UFLA285) for the second one, the result was similar to the combination and different from the control. The UFLA285 treatment alone remained resulted in an effect similar to the control and chemical control in the first year and overcame the control in the second by 26%. Finally, MEN2 showed a detrimental effect in the first year with a 12% reduction in

germination and a beneficial one in the second year by increasing the germination by 17% (Table 5).

Although damping-off caused by *R. solani* has been reported as occurring mainly in pre-emergence (Kirkpatrik & Rockroth, 2001), it is possible that not all fallen seedlings would be a result of *R. solani* infection and a post-emergence damping-off in the field was observed in both years and thus it was recorded (Table 5). In the first year, the number of damping-off seedlings was about the same as in the second but, since it was calculated in terms of damping-off percent and the denominator was higher in the first year, it was expected that the post-emergence damping-off would be higher in the second year. In the first year, both strains alone or in combination reduced damping-off to a similar degree (55-62%), whereas the fungicide combination was different from the control and acted at a much lower degree (14%). In the second year no difference was observed between treatments and control.

Since a difference in shoot dry weight was observed when infected seeds were treated with the antagonists, this effect was checked if also occurring in the field (Table 5). UFLA285 alone induced a higher shoot dry matter than MEN2 or the combination but similar to the control or fungicide. The fungicide and control means were similar to the mixture and higher than MEN2. The mixture was similar to MEN2.

6 DISCUSSION

Seed-borne and seed transmitted diseases are an important trend in cotton production. At early seedling development, a 27% estimated loss in the growers revenue is due to damping-off (Kirkpatrik & Rockroth, 2001) and an uncalculated higher loss as a function of the seed-transmitted diseases, leading to the introduction of pathogens to areas where it had not been previously found (Machado, 2000).

Among 368 strains obtained from research centers, isolated from soil and endophyte from roots, two of them consistently reduced damping-off and bacterial blight on seedlings.

Although breeding lines have some degree of resistance to ramulose (Nascimento et al., 2006), they are not yet available to growers and the disease control relies on fungicide seed treatment and plant sprays throughout the plant cycle (Suassuna & Coutinho, 2007). In drastic seed sanitization by using sodium hypochloride, Soave et al. (1984) observed a reduction in 45% of the disease, which was the reduction mediated by UFLA285 treatment, an even higher reduction was obtained by treating seeds with MEN2 (59%).

The use of commercial seed treatments result in high germination and low post-emergence damping-off, as recently confirmed by Chitarra et al. (2008). However, the germination for the untreated inoculated control found those authors (89%) was much lower than the one obtained in our experiment (53%) (Table 2), which is an indication of the high inoculum pressure obtained by the water restriction method for *Cgc* inoculation. Nevertheless, the germination obtained by the biocontrol agents (MEN2 and UFLA285) was similar to the untreated non-inoculated control (80%).

In the field, the germination for the water treated control in the second cropping season was similar (48%) (Table5) to the one the screening experiment (53%) (Table 2) and in both cases the pathogen each strain when used alone of in combination for seed treated assured germination significatively higher than the water-treated control.

For the first time, the rhizobacterial-based biological control of *Colletotrichum gossypii* var. *cephalosporioides*-mediated damping-off or a simultaneous screening for both bacterial and fungal cotton pathogens by seed treatment of infected seed and a subsequent consistent improvement of germination in the field has been addressed. The screening strategy represented a second chance in the search for the best rhizobacteria for disease control that for some reason would not have had an acceptable disease control for one pathogen.

The other pathogen addressed in the screening was bacterial blight. A seed treatment effective in the control of this disease has been reported either based on a biocontrol agent (Arya & Parashar, 2002; Sbacheiro et al., 2007) or fungicide (Mehta et al., 2005). The only integrated approach for cotton seed treatment, where a same strategy would be effective against both tested diseases has been addressed by Mehta et al. (2005). They found that tolyfluoanid, a fungicide already used in cotton treatment against *C. gossypii* was able to reduce the disease in up to 80%, but the application technology is based on overnight soaking of seeds in the fungicide suspension which is yet to be optimized for large scale use. Besides, since the bacterium does not affect seed vigor (Medeiros et al., 2007), germination was not assessed as a variable to measure the effect of bacterial blight control.

Although most cultivars presently are resistant to the disease, a highly virulent strain (HVS) has already been detected in Africa in 1988, fortunately it has not been reported elsewhere but has been reported as able to overcome

resistance in all commercial cotton cultivars (Chakrabarty et al., 1997) and developing strategies effective in the control of presently relevant diseases and able to face eventual outbreaks of potential menaces such as HVS *X. axonopodis* pv. *malvacearum* in the future are part of a sustainable strategy. Presently, the control of bacterial blight relies on resistant cultivars and copper-based fungicides to hinder eventual epidemic outbreaks in susceptible cultivars.

After treatment, the rhizobacterium colonizes seeds externally and internally and gradually colonizes roots (Huang et al., 2008). Since antimicrobial compounds either are delivered within the bacterial suspension or produced within bacterial development on the seed coat and can lead to a reduction in fungi associated to the seed such as the one observed in the present study. While no pathogenic species was recovered, even the non pathogenic fungi at high population in the seed have been reported as causing detrimental effects on germination and seedling development (McGee, 1995). However, those pathogens have not been related to post-emergence damping-off and the observed fallen seedlings as well as the fungal population below control levels detected (maximum 17%) (Table 4) and the absence of pathogenic species on the blotter test, suggest that the pathogenic fungi was associated to saprofitic growth on organic matter, volunteer plants or seedlings or overwintering resistance structures in the soil such as slerotia and clamidosporia of *Rhizoctonia solani* (Kirkpatrick & Rockroth, 2001).

The recovered bacteria was within the range of the reported as necessary to exert any benefit 10^3ufc/g (Keinath et al., 2000) but the presence of bacteria associated to the untreated control suggest that future works should consider transforming, either by using a selective marker or auxotroph mutants of the antagonists to track their survival on seeds, as previously described (Benizri et al., 2001; Baudoin et al., 2002).

In the field trial, both germination and post-emergence damping-off were obtained from data collected the same day (15 days after sowing), i.e., the germination is a result not only of pre- but also post-emergence (Keinath et al., 2000). By analyzing the post-emergence damping-off in the first year, it was possible to notice that the UFLA285-mediated disease control occurred only after germination while the germination by itself remained constant. Although a similar post-emergence damping-off control was observed for MEN2, a detrimental reduction in germination was observed. In both cases, evidence suggests that induced systemic resistance be part of the underlying disease control mechanisms and from microarray results, UFLA285 was found to differentially regulate 215 genes, many of which are related to the jasmonate pathway defense system (Medeiros et al., in press).

The strains used individually did not consistently improved germination, compared to the combination or fungicide treatment and this result was not unexpected, since Hagedorn et al. (1993) used bacterial inoculants individually but did not observe consistent control of *Pythium* spp and *Rhizoctonia solani* damping-off. However when combining strains, Jetiyanon & Kloepper (2002) obtained control of southern tomato blight (*Sclerotium rolfsii*), pepper anthracnose (*Colletotrichum gleosporioides*) and mosaic in cucumber (*Cucumber mosaic virus*) in two growing seasons.

Here, the observed consistent disease control in both years of the experiment (Table 5), while a control mediated by the bacterial strains alone was not observed, is indicative of a sinergistic effect of the combination. In the field, the pre-emergence damping-off could not be tested but the fallen seedlings after germination at 15 days after sowing were isolated and the only pathogenic fungi recovered from them was *Rhizoctonia solani* (data not presented). In spite of the use of insecticides as seed and in-furrow treatments,

an incidence of cotton borer (*Elasmopalpus lignosellus*) (Santos, 2001) in both years was observed but was not recorded as damping-off.

The broad spectrum activity of the mixture was not tested for other hosts and/or pathogens but it has been previously reported that MEN2 is effective on the control of melon bacterial blotch (Medeiros et al., 2003).

Furthermore, the reduction in fungi recovered from seeds killed by herbicite in the blotter test, suggested that in the absence or presence of reduced level of root exsudates, bacteria associated or not to bacterial born metabolites, suppressed fungal growth and thus initial fungal inoculums, eventually even the those encountered in the soil. This is particularly true because the seeds used in the fungal and bacterial recovery experiment were the same used for planting and, the pre-emergence damping-off may be a result of a combination of different pathogens not necessarily associated to pathogenicity (Howell, 2002).

For the shoot dry weight experiment, data from the second growing season was not recorded since patchy damping-off outbreaks resulted in highly variable diseased plant development due to the variable plant density in the rows, leading to inconsistent results, for instance a higher dry weight for the untreated control since a lower plant density occurred. From recorded data, no significant improvement was observed in spite of the tendency of UFLA285 to overcome the other treatments (Table 5) or detrimental effect from the mixture, since no difference was observed between it and the untreated control or fungicide. Two studies are being presently carried out to formulate the bacterial strains for even higher performance and plant protection of early occurring diseases and also to estimate final yield as a result of seed treatment and/or plant sprays.

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CHAPTER 3:

Transcriptomic analysis reveals simultaneous soil bacterium biotic and abiotic stress alleviation and classical induced systemic resistance.

1 ABSTRACT

Rhizobacteria confer resistance to plant diseases and tolerance to drought. For a broad view of plant microbe interactions the microarray technique has proven to be a powerful tool. Therefore, this work addressed the study of plant responses to a *Bacillus subtilis,* screened for the control of seed-borne diseases, by microarray and addressed osmorregulation activity. The bacterium proved to be efficient in the control of cotton damping-off (*Rhizoctonia solani* AG4), the disease control response occurred when plants were inoculated 9 days after sowing (DAS) and the expression of genes coding for ethylene inducible protein as well as peroxidase were up-regulated in both roots and stems at 13 DAS on rhizobacterium-treated over untreated control plants. Microarray results revealed 246 genes with changed regulation, among which typical jasmonate/ethylene-mediated induction of resistance as well as proline synthesis and aquaporin, both reported as osmorregulation-related genes. Proline was found to accumulate on diseased tissue and this accumulation was higher in treated plants. Aquaporin was up-regulated on treated non stressed plants and down-regulated on treated infected ones, a possible explanation to avoid water drain to infected tissue. The rhizobacterium treatment fosters the plant rapid recovery from a drought stress, results inferred from photosynthesis and shoot dry weight measurements. Treated non-stressed plants maintains longer than untreated. For the first time, the dual role of simultaneously facing biotic and abiotic stresses has been reported and shed light into the exploration of osmorregulation as a novel rhizobacterium-mediated disease control mechanism.

Key words: *Gossypium hirsutum,* ISR, Drought Tolerance, PAL, JA, RT-PCR

2 RESUMO

Rizobactérias conferem resistência a doenças de plantas e tolerância ‘a seca. Para uma visão ampladas interações planta-micróbio, a técnica de microarranjo provou ser uma potente ferramenta. Portanto, este trabalho abordou o estudo das respostas de plantas a *Bacillus subtilis,* selecionado para o controle de doenças cujos agentes etiológicos são transmitidos por sementes, por microarranjo e foi abordada a capacidade de osmorregulação. A bactéria provou ser eficiente no controle do tombamento do algodoeiro (*Rhizoctonia solani* AG4), a resposta de controle da doença ocorreu quando as plantas foram inoculadas 9 dias após o plantio (DAP) e os genes que conferem para a síntese da proteína induzida pelo etileno e para a peroxidase foram expressos tanto em raízes quanto em caules aos 13 DAP. Os resultados de microarranjo revelaram 246 genes com regulação mudada, dentre os quais os relacionados à rota de indução de resistência sistêmica via jasmonato/etileno assim como um relacionado à síntese de prolina e aquaporina, respostas relacionadas à osmorregulação. A proline acumulou-se em tecidos doentes e este foi maior em plantas tratadas. A aquaporina foi super-expressa em plantas tratadas não submetidas a qualquer estresse e sub-expresso naquelas infectadas, uma possível explicação é a de evitar o dreno de água para o tecido infectado. O tratamento com a rhizobacteria garante o mais rápido reestabelecimento de plantas submetidas a estresse hídrico, quando se mediu a fotossíntese e o peso seco de plantas e manteve a fotossíntese por mais tempo ativa em plantas não submetidas a estresse. Pela primeira vez, o papel desempenhado por uma rizbactéria de proporcianar o controle de doença e aumentar a tolerância ao estresse hídrico e a osmorregulação parece ser um novo mecanismos de ação de rizobactérias no controle de doenças de plantas.

Palavras chave: *Gossypium hirsutum,* ISR, Tolerância à seca, PAL, JA, RT-PCR

3 INTRODUCTION

Soil microbes interact with each other and with plants in a series of different ecological relationships (Malik et al., 2005). Cotton plants (*Gossypium hirsutum*) and their rhizospheric soil microbes are no exception, especially because of the continuous cotton planting system with a recent but still inexpressive initiative of rotation systems (Hulugalle & Scott, 2008). Hence, microbes are likely to co-evolve with the cotton plants, no rarely in a detrimental way. Among soil-borne fungi, several have been reported as causing damping-off and wilting to cotton seedlings with estimated losses of up to 27% (Kirkpatrick & Rothrock, 2001). The most common is *Rhizoctonia solani,* the pathogen efficiently infects cotton plants and overwinters as dormant structures (sclerotia, clamidospores) or saprofitical growth on decaying organic matter (Manian & Manibhushanrao, 1990). Eventually plants may overcome the infection but the pathogen builds up its soil population leading to a later outbreak under favorable conditions. Pathogens interfering in the xylem flow simulate a water deficit stress (Dowd et al., 2004) making the plant more susceptible to an eventual drought or salt stress. A constant effort is addressed to breed cotton cultivars able to face abiotic stresses (Parida et al., 2008) but few include the soil-borne pathogen resistance (Lopez-Lavalle et al., 2007).

Other soil inhabiting microorganisms are bacteria referred to as plant growth promoting rhizobacteria (Kloepper et al., 1992). They have been reported for the control of soil-borne pathogens in a wide range of plant species (Mondal & Verma, 2002) and have recently been shown to induce salt tolerance by the down-regulating the sodium transporter (HKT1) in *Arabidopsis thaliana* (Zhang et al., 2008). Another soil bacterium has been shown to induce drought

and salt stress tolerance in the same plant (Cho et al., 2008) and the key molecule involved in this induction (2,3-butanediol) has already been shown to induce systemic resistance in *A. thaliana* against *Pectobacterium carotovorum* subsp. *carotovorum* (Ryu et al., 2005), a common soil-borne pathogen.

In order to explain all gene expression changes mediated by a certain treatment, researchers have been using currently using microarray. The technique allowed Zhang et al. (2007) to explain that observed growth promotion due to an auxin homeostasis after *Arabidopsis thaliana* are exposed to rhizobacterium-borne volatile organic chemicals. The technique has been used in cotton by Dowd et al. (2004) to explain changes after infection by *Fusarium oxysporum* f.sp. *vasinfectum,* a xylem flow interfering pathogen, demonstrating the presence of disease resistance as well as drought stress tolerance gene over-expression. The cotton microarray chip has been updated based on all deposited *Gossypium* spp ESTs (Udall et al., 2007) but no new work has since been published on the plant pathogen interaction and none has addressed the triple interaction (rhizobacterium, pathogen, *G. hirsutum*).

To probe plant-signaling pathways activated by cotton seed treatment with *Bacillus subtilis* UFLA285 (Medeiros et al., 2008) that mediate damping-off control, we have characterized the overall transcriptomic change using the most recent developed *Gossypium* spp microarray chip. Results showed a series of defense-related as well as stress tolerance genes. In this study we report that bacterial treatment induce a typical jasmonate/ethylene defense signaling pathway, cell wall reinforcement as well as typical drought stress alleviation with the regulation of genes and accumulation of proline in an osmoregulation-related manner. The PGPR treatment was able to more rapidly reestablish the net photosynthesis after a drought stress, maintain it when on diseased plants and for a longer period under no stress.

4 MATERIALS AND METHODS

4.1 Bacterial, plant and fungal cultures

Bacillus subtilis UFLA285 previously selected for the control of cotton seed-borne diseases (Medeiros et al., 2008) was streaked from -80ºC preserved slants to LB agar and after 24h incubation at 28ºC isolated colonies were transferred to 250mL-erlenmeyers containing 50mL of LB. After 24h growth in orbital shaker at 250RPM at the same temperature, bacterial cells were harvested by transferring the bacterial growth to 2mL microfuge tubes and centrifugation at 10,000G for 5min. Cells were ressuspended in saline buffer (0.85% NaCl) and concention set for 10^9 cfu/mL by reading the suspension optical density (0.7 absorbance) in spectrophotometer at 600nm, inferred from a standard curve (data not shown).

Gossypium hirsutum seeds cv Deltapine Acala 90 were surface sterilized in sodium hypochloride (0.5% active chloride), washed thoroughly with sterilized distilled water, air dried and then treated with the bacterial suspension (2mL 10^9 cfu/mL/g of seed), water (2mL saline buffer/g of seed) or fungicide (triadimenol 10µg active ingredient/g of seed) and sown into 2L pots containing 400g of the potting mix Sunshine® All-Purpose Planting Mix (Sun Gro Horticulture, Vancouver, CA), fertilized with 5g of Osmocote fertilizer (Scotts-Sierra Horticulture, Marysville, OH, USA), irrigated to field capacity daily. Planted pots were kept in a growth room under controlled temperature (25ºC±4), relative humidity 40±10% and light (200µmol.$m^{-2}s^{-1}$) by using a combination of metal halide and high sodium pressure lamps set for 14h/day.

The pathogen used, *Rhizoctonia solani* AG4 To prepare the pathogen suspension (Strain 1, Dr. Wheeler's Collection, TAES, Texas A&M) was initially deep freezer preserved as sclerotia in glycerol (40% in distilled water

v/v) were transferred to potato dextrose agar (Difco Laboratories, Detroit, MI, USA) and after a 7-day incubation at 24ºC, 5 mycelium plugs from the extremity of the colony were inoculated into 500mL-erlenmeyers containing 100mL V8 medium (200mL commercial V8 juice and 3g $CaCO_3$ per liter). The erlenmeyers were incubated in orbital shakers at 150 RPM, 24ºC and agitated for 4 days, when the mycelium was harvested by centrifugation, resuspended in water and macerated in a blender to give a homogenous suspension. The threshold concentration to inoculate plants in the experiments was determined by inoculating plants at different concentrations. A 10^2 cfu/mL was determined as the minimum concentration to cause wilting in all plants 4 days after inoculation and was the one used in all trials where inoculation was performed. Unless differently mentioned, at 9 days after sowing, plants were inoculated with a drop of 250µL of a 10^2 cfu/mL of the pathogen suspension.

4.2 Time necessary for damping-off resistance response

Although elicited cotton plants require four days after treatment to be able to control damping off (Jabaji-Hare & Neate, 2001), *Rhizoctonia solani* is a soil-borne pathogen and as such seedlings may have to face the pathogen upon germination. However, cotton seeds were treated with the benefitial bacterium and challenged with the pathogen upon germination (5 days after sowing), 6, 7, 8 or 9 days after sowing (Figure 1C). From the 4^{th} to the 10^{th} day after each inoculation time, plants were assessed daily for the disease severity according to a 1-5 numerical scale previously described (Keinath et al., 2000), where (1) represents no visible symptom, (2) a few pinpoint lesions on diffuse discolored areas, (3) distinct necrotic lesions, (4) girdling lesions and (5) damped-off or killed seedling. The obtained data was analyzed collectively by the area under the disease progress curve (Shaner & Finney, 1977).

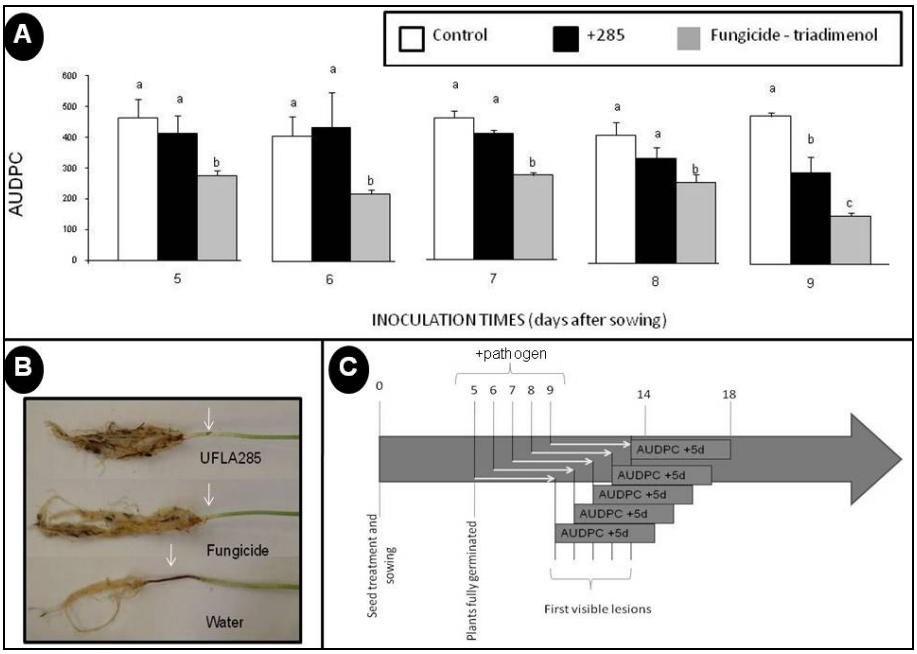

FIGURE 1 Screening for the time necessary for the bacterial treated plant response to infection. (A) Seed treatment with *Bacillus subtilis* UFLA285 (+285) protects cotton plants when challanged nine days after sowing, fungicide treatment controls the disease regardless of the inoculation time. Treatments were compared based on the area under the disease progress curve (AUDPC) (Shanner & Finney, 1977) over a 9-day period from inoculation. Data followed by the same letter in each period are not different by Tukey's test $P \leq 0.05$; (B) 17-day old cotton plants, inoculated at 9 days after sowing with arrows showing brownish necrotic lesions in the root/shoot interphase typical symptoms of *Rhizoctonia solani* infection; (C) Seeds were treated with the rhizobacterium, fungicide (triadimenol) or water and inoculated with *R. solani* AG4 (+pathogen) at 5, 6, 7, 8 or 9 days after sowing, from the 4th to the 9th

4.3 Plant sampling and RNA extraction

Since at four days after inoculation the first necrotic lesions were visible (Fig 1C), mRNA expression was likely to occur at an earlier time point and an experiment was carried out following the inoculation scheme determined in the previous experiment. Seeds were treated with the biocontrol agent or water, seedlings inoculated and plant parts sampled following the described in drawn timeline (Fig 2B). At 10, 11, 12 and 13 days after sowing, stem and root plant parts, infected with the pathogen (Kirkpatrick & Rothrock, 2001) were harvested. Those parts are the ones more commonly reported as infected by the pathogen and thus leaves were not considered in the experiments.

The harvested plant parts were quickly processed (<5min per sample) by separating the plant from the soil and splitting it to roots and shoots, washing them under tap water, wrapping in aluminum foil, labeling, freezing in liquid nitrogen and storing them at -80ºC until RNA extraction.

Samples were ground in mortar and pestle under liquid nitrogen and RNA extracted following the "hot borate" protocol (Wan & Wilkins, 1994) adapted for micro-scale extraction (0.2g of macerated fresh tissue). The RNA was purified using the RNEasy MinElute Cleanup kit (Qiagen, Valencia, CA, USA) including the RNase-free DNAse treatment step from the same manufacturer. The clean RNA was quantified and stored at -80ºC until use.

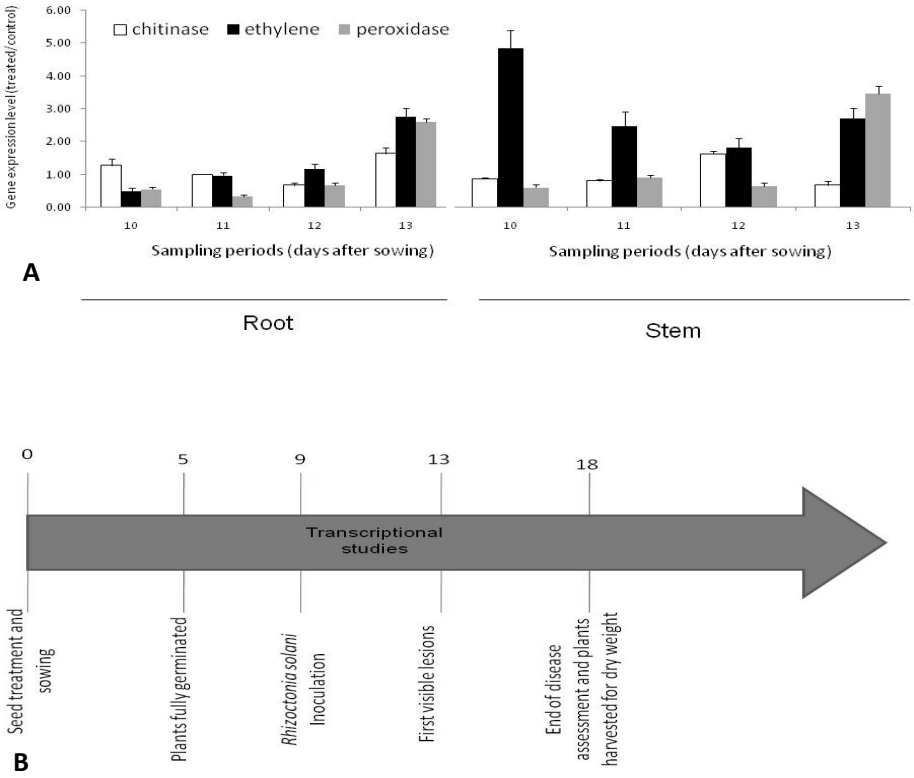

FIGURE 2 Study of the expression level of chitinase, ethylene inducible protein and peroxidase. (A) The seed treatment with *Bacillus subtilis* UFLA285 induces the expression of ethylene and peroxidase in both stem and root at 13 days after sowing through the gene mRNA RT-PCR; (B) Pathosystem operative mode as determined on the first experiment (*Rhizoctonia solani* AG4 and *Gossypium hirsutum* DP-90) after seed treatment with *B. subtilis* UFLA285 for the screening of the best time after inoculation for microarray analysis.

4.4 RT-PCR of induced resistance marker genes and validation of microarray result

First strand cDNA was synthesized from 5µg of total RNA following methodology previously described (Zhang et al., 2007) and PCR performed using the (5'-3') primers, designed based on deposited sequences (Table 1).

For the microarray result validation, from the ones with significatively changed expression, six genes were randomly chosen (Table 1) among the ones that were found with changed regulation and the primers designed based on the UNIGENE used to generate each microarray probe (Comparative Evolutionary Genomics of Cotton, available at http://cotton.agtec.uga.edu/ProbePortal).

For both experiments, agarose gel electrophoresis images were taken by Kodak Gel Logic 100 Imaging System (Fisher ScientiWc, Houston, TX, USA) and the band intensity quantified by Image J 1.33u (http://rsb.info.nih.gov/ij/, National Institute of Health, USA).

TABLE 1 Details of primers used in the resistance induction time course and validation of microarray results

Gene putative function	EST Code	Forward primer	Reverse primer
Endochitinase	CD486396	ATGGAGCTGCTGGCGATGGTATAA	TTGATTGCTTTCTGCTCGGCACAG
Ehylene inducible protein	CD486177	GGCGCAATAGCTGAAACCCACAAA	ACCCACAGACGAAAGGAATCCGAA
Peroxidase	CD485924	TGGTGCCAGTCTCATCATGCTTCA	ATGTTGGTGTTAAGCGCCACACTG
Housekeeping Polyubiquitin	CK738219	GACACCATTGACAACGTCAAGGCA	AAGACGCAAGACAAGGTGGAGAGT
Aquaporin	BG443217	GCCGAATTCATCGCTACTCTCCTT	AACATCAACCCAAATGTCTCCGCC
Ethylene binding protein	CO104019	ATGAACCGATACCCGAGGTTTCCA	AAGGTTCCCAACCAGATCCGTGAA
Heat shock	DV848869	TTCCTCCCTAAATCCATCCACGCT	TACCAGCACTGATCGGTTTCCCAT
Lipoxygenase	BF278101	AACCGTAACGTCTAGGCAGGGTTT	TTCAGAAAGCGGCTTACCGGGATA
Cytochrome P450	Cotton12_10944_01	CATCAAAGGGCTTATGCTGGTCCT	ACATGCCCTCCTTCCTAACCCAAA
Xyloglucan endoglycosyl transferase	BF271751	TTTCTGTCGCTTCCATGGCTGTCT	TGTGGTATTGCCCAGGAACTCGAA

4.5 Microarray analysis

For each time set, microarray hybridizations consisted of four biological replicates, one of which consisted of a dye swapping. The previously obtained target RNA was transcribed to aRNA in a three step transcription using Amino-Allyl aRNA Amplification Kit (Ambion, Austin, TX, USA) and labeled with NHS dyes Cy3 or Cy5 (Amersham Biosciences, Little Chalfont Buckinghamshire, UK) according to the manufacturer's protocol.

Sixty-mer oligonucleotide microarray slides containing 22,787 oligonucleotide probles were obtained from the joint project University of Georgia/Iowa State University/University of Arizona (Udall et al., 2007) and a list of probes can be accessed at the manufacturer's website (http://cotton.agtec.uga.edu). Slide pre-hybridization was performed according to the manufacturer, whereas hybridization and post-hybridization followed the *Arabidopsis thaliana* protocol (Zhang et al., 2007). The arrays were scanned using a GenePix 4100 array scanner (Axon Instruments, Sunnyvale, CA, USA). Spot statistical analysis was performed according to the manufacturer's guidelines (Gene-Spring 7.0; Silicon Genetics, Redwood, CA, USA). A 40% change, either up- or down-regulation, in the expression level compared with the control was selected as the threshold for a gene to be classified as altered in response to rhizobacterium treatment. Only genes that passed the Flag Filtering, identified as present (Gene-Spring 7.0), and passed the T-test P-values 0.10 were considered differentially regulated with the rhizobacerium treatment.

4.6 Proline abundance analysis and aquaporin expresion

Seeds were treated with the rhizobacterium or water and submitted to three stress conditions: (no stress) plants irrigated at field capacity, (-H_2O) not irrigated from the 9^{th} to the 13^{th} day after sowing (DAS) or inoculated at the 9^{th} DAS. In all cases, stems were sampled at 13 DAS, stored and ground similar to

the procedure described for RNA extraction. Stems were the plant part chosen for the analysis since the microarray experiment was based on this plant part.

For proline analysis, a standard curve determined the relationship (y=12.1x) between proline concentration ("x" 0-0.15g proline/L) and absorbance at 520nm (y) to estimate the proline abundance in the plant tissue. The stem proline abundance was estimated by weighing 0.25g of ground tissue and transferring to a 2mL-microfuge tube containing 1.2mL of 3% aqueous sulfosalicilic acid solution and vigorously agitating in vortex for 1min to simultaneously thaw the sample and release the proline from the plant tissue into the solution. The tubes were subsequently centrifuged at 10,000G for 10min and 0.5mL of the supernatant was transferred to a new 2mL microfuge tube and diluted 1:1 with 0.5mL aqueous solution. The 1mL resulting solution was transferred to 10mL screw cap glass tubes, mixed with 1mL acid ninhydrin, prepared according to Bates (1973) and 1mL glacial acetic acid. The reagents were mixed by inversion and tubes warmed to 100ºC for 1h in the absence of light. Tubes were then quickly cooled in an ice bath. The reaction mixture was extracted by adding 2mL of toluene and vigorous agitation in vortex for 20s. The chromophore containing toluene (upper phase) was transferred to new test tubes and optical density measured at 520nm in spectrophotometer. Abundance of proline was calculated as μg of proline/g stem tissue.

For aquaporin expression, 0.2g of ground tissue was used for RNA extraction, first strand cDNA synthesis and PCR as previously described. The aquaporin band intensity measured from the gel image for each treatment combination was analyzed as described in the statistics section.

4.7 Photosynthesis measurements and plant dry weight

Cotton seeds were treated with the PGPR or water and submitted to the three stress conditions mentioned above, changing however the number of days after inoculation (inoculated) or no irrigation (-H_2O) to 8 days instead of 4 in order to both allow the first true leaf to be fully expanded in all treatments and assess phenotypic differences inferred from the transcriptomic's study at an earlier time point. Photosynthesis was assessed at the last day (8^{th}) of the stress condition (17 days after sowing).

In order to assess the plant recovery after water stress, plants were irrigated after the 8-day without irrigation and photosynthesis was measured 24h afterwards. In both assessments, photosynthetic measurements were made using LI-COR 6400 portable photosynthesis systems (LI-COR Biosciences, Lincoln, NE) with steady CO_2 load (380 Pa) and light intensity (2000µmol.m^{-2}.s^{-1}) and desiccant tube in bypass mode.

After the last photosynthesis measurement, plants were watered daily to field capacity and 25 days after sowing, they were sampled and shoots dried in an oven at 70^{o}C for three days and the weight for each treatment was recorded.

4.8 Statistical analysis

Whenever applicable, plots were composed by four plants. Those plants were either pooled for RNA extraction or the measured severity and photosynthesis averaged and each experiment was composed in order to reduce errors and the experiments encompassed three biological replicates. The obtained data was submitted to variance analysis ANAVA and for significant effects ($P<0.05$), means were compared according to Tukey's test using the SAS software (SAS Institute, Cary, NC, USA).

5 RESULTS

Damping-off is a worldwide cotton disease that affects plants at early seedling development and whose control relies on fungicide seed treatment. To foster long-term sustainability of cotton over 300 plant growth promoting rhizobacteria were screened for damping-off (Medeiros et al., 2008). The reduction in the area under the disease progress curve (AUDPC) was observed when plants where treated with *Bacillus subtilis* UFLA285 and inoculated 4 days after germination (Figure 1).

The requirement of a time for both the establishment of the rhizobacterium and the response to the disease are indications of the induction of plant systemic resistance genes (Hammerschmidt & Kuc, 1995). The expression of those genes is assumed to occur before visual symptoms of the disease are observed. Hence, cotton plants coated with *B. subtilis* UFLA285 were inoculated with the pathogen 9 days after sowing (4 days after germination) and monitored for the expression level of ethylene inducible protein, peroxidase, endochitinase and the housekeeping polyubiquitin, both stem and roots of infected plants either treated with UFLA285 or water from the 10^{th} to the 13^{th} day after sowing (DAS) (Figure 2). The genes being selected on the basis of the peculiar induced systemic response pathway it represents, i.e., chitinase as a marker for the salicylic acid pathway, ethylene inducible protein as a marker for the jasmonate/ethylene pathway and peroxidase as a scavendger reported as operative in both induced resistance pathways (Schenk et al., 2000).

In stems, the level of ethylene inducible protein was consistently induced (1.83-4.84 fold change) throughout the sampled time frame, while in roots it was initially down-regulated (0.50 fold change at 10DAS) then

remained unchanged (0.97 and 1.17) until 13DAS when it was upregulated (2.76 fold change) (Figure 2).

The level of peroxidase was initially down-regulated (0.34-0.56 for roots at 10-11 DAS and 0.61 for stems at 10DAS), then unchanged (0.68 for roots at 12 and 0.68-0.91 at 11-12 DAS) and up-regulated at 13DAS for both plant tissues (2.61-3.47 fold change for root and stem respectively) (Figure 2).

Similarly, at one single time point (1.64 fold change at 13DAS for roots and 1.63 fold change at 12DAS for stems) chitinase was found to be up-regulated (Figure 2).

At 13 DAS, light brown lesions in the borders and necrosis in the center, tipical of *R. solani* infection (Kirkpatrick & Rothrock, 2001) were easily visible in the water control and to a lesser extent in the 285 treated plants, comparable to the fungicide (triadimenol) treated plants (Figure 1 B). At that sampling time the fungal could be recovered from all inoculated seedlings from plating the root/shoot interphase on potato dextrose agar amended with streptomycin (100ppm).

In order to study the overall gene expression, labeled mRNA from stem tissue harvested at the last sampled time point (13^{th} days after sowing) was hybridized with microarray slides, designed for over 22,000 ESTs (Udall et al., 2007). A total of 247 genes were differentially expressed with 285 treatment over water control both treatments challenged with *R solani* inoculation. Microarray responses were validated by RT-PCR analysis of selected genes. All five genes tested showed a similar fold change (Figure 3).

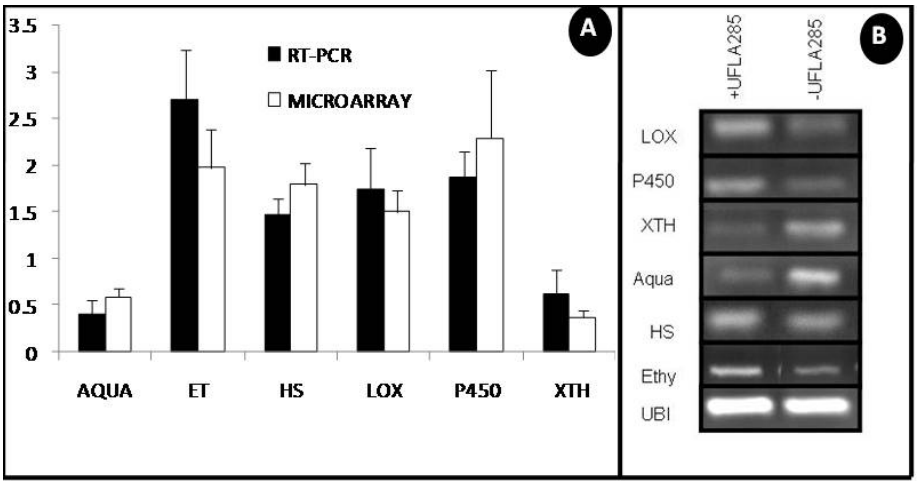

FIGURE 3 Expression level of selected genes. Expression level from microarray data and RT-PCR. (A) expression level of each gene measured as the band intensity of the gel and (B) gel for each gene aquaporin. Gene codes: aquaporin (AQUA), xyloglucan endoglycosylase (XTH), cytochrome P450 (P450), ethylene response factor (ET), heat shock protein (HS), ubiquitin (UBI).

Among the putatively known genes, most of which were up-regulated, the largest group (12% of total) was associated with defense responses, all of which but aquaporin and dehydroascorbate peroxidase where up-regulated (fold change >1.4 and pvalue<0.05). The defense genes where separated in anti-oxidant/scavengers, PR-proteins, jasmonic acid biosynthesis, phenylpropanoid pathway and osmorregulation (Table 3).

A total of six genes were associated with cell wall modulation, those associated with reinforcement (transferase, callose synthase and lipid transfer protein) were up-regulated whereas those associated with loosening (xyloglucan hydrolase) were consistently down-regulated.

Some genes where even related to stress alleviation (alcohol dehydrogenase, heat shock, luminal binding protein, protein disulfide isomerase) most of which (67%) were up-regulated. Another large group of genes found to be up-regulated was the signal transduction (13% of total genes) and a similar scenario was noted for the transcription factors (Table 3).

A different set of gene classes found to have significatively changed regulation was that associated with the primary metabolism. The genes coding for the primary metabolism found to be up-regulated were divided into six categories: those associated to the metabolism of macromolecules (protein, lipid and protein), those associated with replication, transport and miscellaneous, among which a set of 77 genes for which no significative homology was found with known genes (Table 2).

TABLE 2 Primary metabolism gene regulation by combined *Bacillus subtilis* UFLA285 (treated) or water (control) and inoculation with *Rhizoctonia solani* AG4 on the 9th-day after sowing. Bold-marked-responses were up-regulated (ration>1.4)

Groups	Representants	Ratio (regulation)	Genes
Lipid metabolism (3.6%)			
Anabolism	**oxysterol binding protein, β-ketoacyl-CoA synthase, Erg-1, sterol-delta-7-reductase**	1.4-1.77 (100% UP)	4
Catabolism	**Acid phosphatase class B, 2-hydroxyphytanoyl-CoA lyase, lipase**	1.52-2.48 (100% UP)	5
Carbohydrate metabolism (3.6%)			
Catabolism	**KHG-KDPG bifunctional aldolase-like, aldehyde lyase, malate dehydrogenase**	1.48-2.77 (100% UP)	5
Dual role	**Dihydrolipoylisine-residue acetyltransferase, phosphoenolpyruvate carboxylase, sucrose (phosphate) synthase*** **	1.40-1.44 (100% UP)	4
Protein metabolism (4.5%)			
Anabolism/modulation	**caleosin, fasciclin**, **asparagines tRNA ligase, ubiquitin protein ligase/hydrolase, Claritin heavy chain,** 60S ribosomal protein BBC1, 30S ribosomal protein	0.36-1.80 (42% UP)	7
Catabolism	Endopeptidase	0.57	1

…Continued…

TABLE 2 Cont.

Replication (8.9%)	**Helicase, rna recognition protein, histone (acetylation), RNA polymerase, small nuclear ribonucleoprotein, C-terminal domain phosphatase-like 1, reverse transcriptase, mini-chromossome maintenance protein, ligase, relA/spo T homologous protein RSH2, ribonuclease**	0.57-2.09 (95% UP)	22
Transport (8.5%)			
Amino acid	**Peptide transporter, aminoacid permease**	1.45- 1.88 (100% UP)	3
Ions	**Intracellular chloride channel,** copper protein, H+-transporting ATP synthase, **sulfate transporter, potassium transporter**	0.33-5.34 (67% UP)	6
Transmembrane	**coatomer protein complex epsin-like protein, exocyst dubunit EX070 family protein E1, importin beta2 subunit family protein (ABC transporter), got1-like family protein, sedlin, transport component particle (TRAPP)**	1.52-2.29 (100% UP)	8
Inespecific			
Other	Mitochondrial phosphate translocator, **mitochondrial carrier protein-like, ureide permease 1, purine permease**	0.33-1.72 (75% UP)	4
Miscellaneous (32%)			
Respiration	**Cytochrome b5 DIF-F, 4-phosphopantothenoylcysteine synthetase**	1.59-1.69 (UP)	1
Flowering	Glicine-rich RNA-binding protein	0.52 (DOWN)	1
Photosynthesis	Light harvesting complex	0.54 (DOWN)	1
Unknown			77

TABLE 3 Secondary metabolism gene regulation by combined *Bacillus subtilis* UFLA285 (treated) or water (control) and inoculation with *Rhizoctonia solani* AG4 on the 9th-day after sowing. Bold-marked-responses were up regulated (ration>1.4)

Gene classes	Response	Ratio (treated/control)	Gene numbers
Defense (12.1%)			
Anti-oxidants/scavengers	**glutathione-S-transferase, peroxidase,** dehydroascorbate redutase, **MATE efflux family protein, thioredoxin, purple acid phosphatase, cytochrome P450**	0.54-2.34 (93% UP)	15
PR-protein	**Thaumatin-like protein, resistance induced protein 13, uncharacterized resistance protein, major cherry allergen, Endo-beta-acetylglucosaminidase**	1.51-2.06 (100% UP)	5
Jasmonic acid biosynthesis	**Lipoxygenase*, allene oxide cyclase**	1.51-1.60 (100% UP)	2
Phenylpropanoid-pathway	**Phenylalanine ammonia-lyase, caffeic acid O-methyltransferease, 2-hydroxyisoflavone reductase, cinnamoyl CoA reductase, dihydroflavonol 4 reductase**	1.64-2.50 (100% UP)	6
Osmorregulation	**Pyrroline-5-carboxylate synthetase**, aquaporin	0.59-1.65 (50% UP)	2
Cell wall modulators (3.6%)			
Reinforcement$$	**Transferase, UDP glycosyl transferase 88B1,** Glycosyl transferase, cellulose synthase**, callose synthase**, **Lipid transfer protein**	0.48-1.2 (67% UP)	6
Loosening	xyloglucan endoglycosyl/hydrolase	0.37-0.58 (100% DOWN)	3

…Continued…

TABLE 3 Cont.

Stress-related (2.4%)	**heat shock protein, alcohol dehydrogenase, protein disulfide isomerase, luminal binding protein** , chaperone protein DNAJ-related	0.55-1.81 (88% UP)	8
Signal transduction (13%)			
Hormone-induced	**Brassinosteroid regulated protein, ethylene receptor, ethylene-induced calmodulin-binding protein, growth factor like protein**	1.43-1.85 (100% UP)	5
Ca^{2+}- dependent kinases	**Calcium dependent protein kinase, calmodulin-binding protein, CDPK adapter protein 1, CBL-interacting protein kinase 21**	1.48-1.53 (100% UP)	5
Leucine-rich repeat	**Leucine rich repeat protein kinases**	0.57-1.92 (75% UP)	4
Lectin repeat	**Lectin-like protein kinase**	1.50-2.06 (100% UP)	2
Serine/threonine kinase	**NIMA-related protein kinase, protein phosphatase 2C, others with no distinct domain**	0.58-2.29 (78% UP)	9
Other	**Avr9/Cf9 rapidily elicited protein, cdk5 regulatory subunit associated protein 3, diacylglycerol kinase, NPK1-related protein kinase, protein phosphatase 2C, rhomboid family protein**	1.42-1.85 (100% UP)	7
Transcription factor (7.3%)			
Hormone-related	**Auxin response factor, ethylene response binding protein, abscisic acid-induced protein**	1.43- 1.98 (100% UP)	4
WRKY-type	**Wound-induced leucine zipper zinc finger (WIZZ)**	1.44-1.62 (100% UP)	2
WD-40	**GhTTG2, other myb-transcr**	1.59-2.07 (100% UP)	4
MYB-like	**Myb transcription factors**	1.68-2.05 (100% UP)	4
Other	**Tfiis domain-containing protein, phavoluta-like HD ZIP III protein, WREBP**	1.50-1.86 (100% UP)	4

The genes targeting replication were all up-regulated: mini-chomossome maintenance protein (DNA replication iniciation), unwinding of nucleic acid (helicase), ribonuclease III and helicase activities (ribonuclease), alternative splicing as well as single-stranded RNA binding (RNA recognition protein), double stranded RNA binding (C-terminal domain phosphatase-like 1), acetylation (histone), RNA replication (RNA polymerase), DNA polymerase (reverse transcriptase), ligase (ARIADNE-like protein), guanine tetraphosphate metabolic process (rel A/spo T homologous protein RSH2), nucleic acid binding (small nuclear ribonucleoprotein).

Another set of genes also involved in the primary metabolism were those associated to transport either of aminoacids (peptide transport and permease) both up-regulated, chloride, potassium and sulfate ions up, whereas H^+ and copper transporters were down-regulated. Also included in the transport but more intrinsically related to the transmembrane nature are related to both citoplasmic membrane selective transport of aminoacid lysine (coatomer protein complex epsin-like protein), inespecific exocytosis (exocyst dubunit EX070 family protein E1), ABC transporter involved in the import of molecules through the membrane envelope (importin beta2 subunit family protein), Golgi complex internal transport (got1-like family protein), endoplasmatic reticulum and Golgi complex-mediated transport (sedlin, transport component particle). Yet some transporters have been included in the "other category" since a few members were present as associated to a similar function. There were those associated with the respiration process in the mitochondria (mitochondrial phosphate translocator and mitochondrial carrier protein-like), replication of genetic material (purine permease) or even transport of a wide variety of heterocyclic nitrogen compounds (ureide permease 1).

The metabolism of macromolecules was analyzed as the pathways of energy generation and alternative compound anabolism (Anapleurotic

reactions) inferred from the genes representing enzymes found to be up-regulated based on a well established pathway (Nelson & Cox, 2002) unless mentioned as described elsewhere (Figure 4).

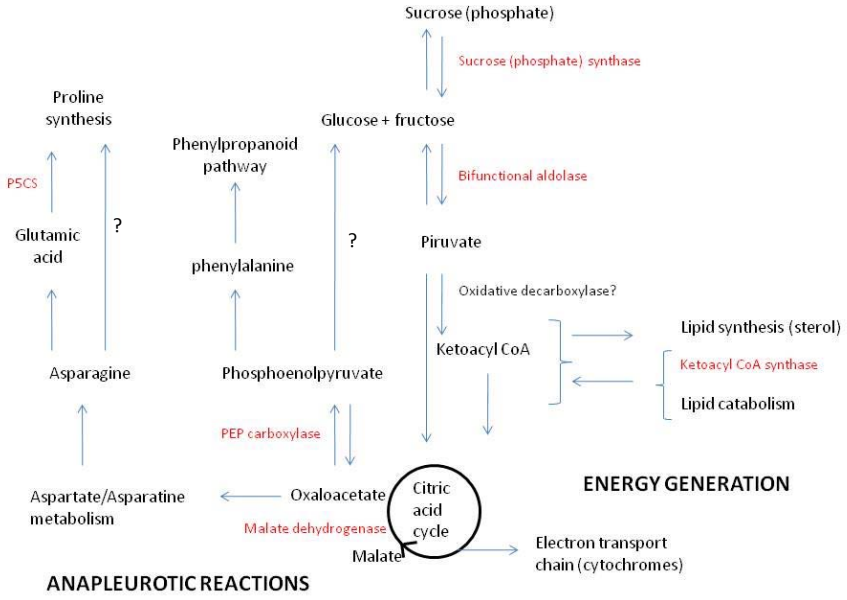

FIGURE 4 Energy generation and anapleurotic reactions likely to be operative, inferred from the microarray results and adapted from previously described schemes (Nelson and Cox, 2002; Delauney & Verma, 1993; Bouter & Barber, 1963). Red font characters denote enzymes found to up-regulated after the rhizobacterium seed treatment

From the disaccharide sucrose, glucose and fructose are converted through a bifunctional aldolase to generate piruvate and is either converted ditectly to oxaloacetate or malate to ketoacyl CoA through oxidative carboxylase, but this enzyme has not been found to be up-regulated in the studied system. However the last product is likely to accumulate in treated plants, since the ketoacylCoA synthase as well as the lipid degradation enzymes (lipase, 2-hydroxyphytanoyl CoA lyase, acid phosphatase class B) are up-regulated resulting as final product the β-ketoacyl-CoA, which in turn may be used in the citric acid cycle to generate energy or in the synthesis of sterol-like compounds (oxysterol binding protein, Erg-1, sterol-delta-7-reductase).

In the citric acid cycle, genes involved in the synthesis of cytochrome (cytochrome b5 DIF-F and 4-phosphopantothenoylcysteine synthetase) suggest an increase in the electron transport chain, the final step in the generation of energy. Another function of the citric acid cycle is the synthesis of compounds such as aminoacids. An enzyme coding for the conversion of malate to oxaloacetate (malate dehydrogenase) is upregulated, thus there is an accumulation of oxaloacetate, a key molecule used in the synthesis of asparagines as well as phosphoenolpyruvate (PEP). The asparagines/aspartate pathway is operative (asparagines tRNA ligase) and is likely to be the substrate for the synthesis of proline, whose synthesis in plants starts either from L-glutamic acid or asparagines (Delauney & Verma, 1993), as shown in Figure 5, and the up-regulation of piroline-5-carboxylate synthetase suggests that the initial substrate for the synthesis of proline in the treated system is the glutamic acid. The main pathway for the synthesis of glutamic acid is in a side pathway in citric acid cycle at the α-ketoglutarate step but no enzyme associated to this conversion is up-regulated. Alternatively, asparagine can be converted to glutamic acid (Barber & Boulter, 1963) then giving rise to proline.

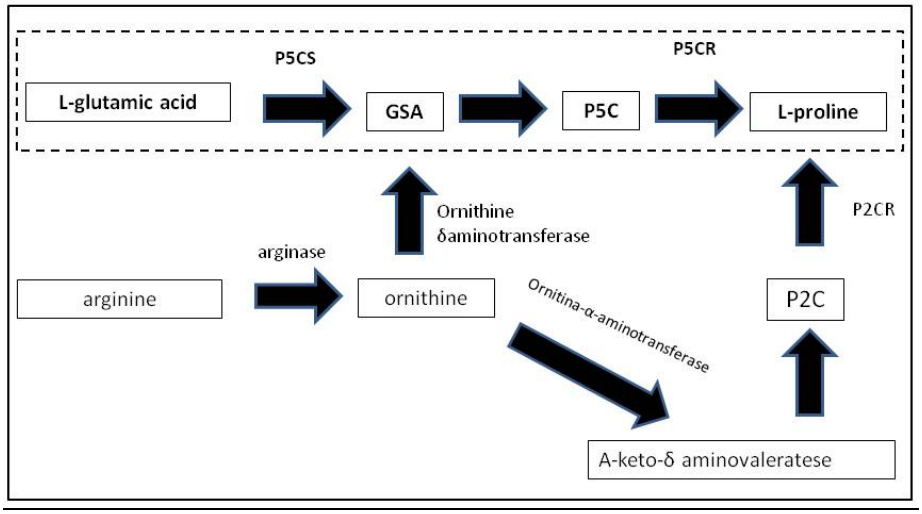

FIGURE 5 Proline synthesis pathway in plants. P5CS: pyrroline-5-carboxylate synthase, GSA: glutamate-□-semialdehyde, P5C:pyrroline-5-carboxylate, P2C:pyrroline-2-carboxylate, P2CR: pyrroline-2-carboxylate reductase. Dashed line delimited area is the pathway likely to be operative in the studied system (adapted from Delauney & Verma, 1993)

The oxaloacetate is converted to phosphoenolpyruvate (PEP) since phosphoenolpyruvate carboxylase gene is up-regulated, in turn PEP can either be converted to amioacids or glucose. The aminoacids such as phenylalanine is an initial step in the phenyl propanoid pathway (Table 3), others are used for the synthesis of ubiquitin (protein modulation for proteossomic degradation), structural component of organelle membranes (clarithin heavy chain) and the stability of the synthesized proteins is assured by the down-regulation of endopeptidase. Yet undetermined is the role of the down-regulation of the translation key proteins (60S ribosomal protein BBC1 and 30S ribosomal protein).

PEP can still be converted to glucose by gluconeogenesis. Actually the most common way of glucose synthesis in C_3 plants, such as cotton, is through photosynthesis by Ribulose 1,5 biphosphate carboxylase/oxidase (RUBISCO)-mediated CO_2 fixation. Cotton genes coding for RUBISCO are present in the microarray chip used (such as Cotton12_00010_02, Cotton12_00189_01, Cotton1200268_02) but none of them showed changed regulation or pvalue below 0.10 (data not presented) and some the NCBI (www.ncbi.nlm.nih.gov) deposited RUBISCO coding genes in cotton (such as DY255474, DT052619, DT047065) are not present in the microarray chip used. In the future, with the availability of fully sequenced *Gossypium hirsutum* genome, we may better understand the regulation of photosynthesis-related genes and their role on glucose synthesis in rhizobacterium-treated plants over water controls.

Glucose which in turn either can be converted back to sucrose or broken down to piruvate and start over the citric acid cycle or simply accumulate in vacuoles. Both the possible accumulation of hexoses (such as glucose) and proline are indications of an osmorregulation phenomenon, commonly found in drought stressed plants (Watanabe et al., 2000). Glucose-related genes have already been reported in plants infected with xylem flow interfering pathogens and its presence was discussed in terms of osmorregulation (Dowd et al., 2004). Another evidence of changes in the plant gene expression as a result of water deficit caused by a pathogen infection was the up-regulation of aquaporin, a protein involved in the cell-to-cell water transport through the membranes (Tyerman et al., 2002).

In an attempt to study the role of *B. subtilis* UFLA285 on osmoregulation, expreriments were carried out to measure the proline abundance and aquaporin expression.

Cotton seeds were treated with *B. subtilis* UFLA285 or water and were either not submitted to any stress (no stress) or not irrigated for 4 days or

inoculated with *R. solani* AG4 and sampled 4 days after inoculation. The first two treatments (no stress and no irrigation) had a similar level of proline whereas the infected plants accumulated proline and this accumulation was even higher in the treated plants (Figure 6).

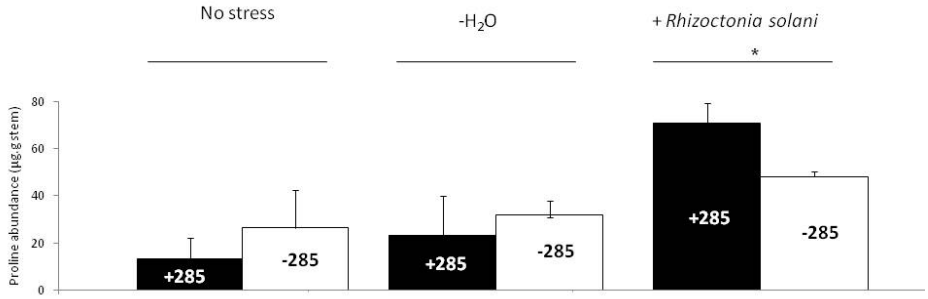

FIGURE 6 Cotton strems four days after inoculation with *Rhizoctonia solani* AG4 accumulates proline more than non-stressed plants (No stress) or submitted to four days without irrigation (-H2O), this effect was more pronounced on *Bacillus subtilis* UFLA285 (+285) treated plants compared to untreated ones (-285).

This level of accumulation of proline in infected plants (up to 80µg/g stem) is comparable to an 8 day drought stress (Chakraborty et al., 2002). In regard to aquaporin, when no stress was applied, this gene was expressed in a higher level on treated plants. To a higher extent on drought stressed plants, however without any difference between treated and control. Conversely, this gene was down-regulated in treated and infected plants compared to untreated and infected (Figure 7).

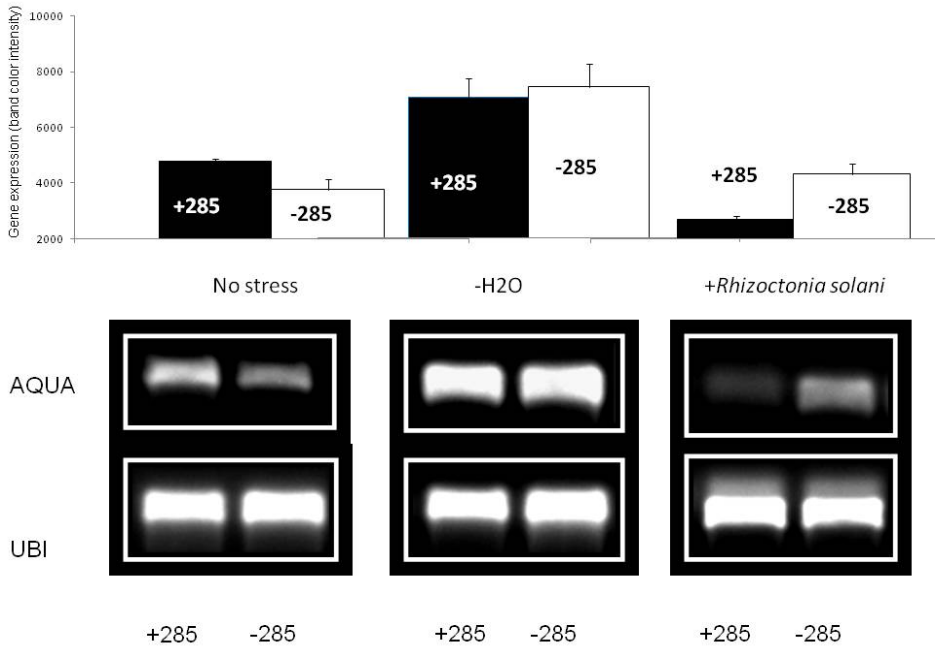

Figure 7 Aquaporin (AQUA) and the housekeeping Ubiquitin (UBI) expression level in rhizobacterium-treated (+285) and untreated (-285) plants under no stress, no irrigation for four days (-H_2O) or infected, four days after inoculation (+*Rhizocotnia solani*). The rhizobacterium treatment increased the aquaporin expression of aquaporin under no stress and reduces it under biotic stress. Under drought stress this gene is up regulated inespecifically in treated (+285) and control (-285) plants.

Considering the detrimental effect of pathogens on plant development and the similarities found between *Rhizoctonia* infection and drought stress, an experiment was carried out where plants were subjected to the same treatments and stress conditions mentioned above, assessing photosynthesis on plants 8 days after inoculation or withholding water in order to observe a down-stream response as a consequence of a gene expression change (Figure 8) and to have

the first fully expanded true leaf for more reproducible measurements based on preliminary tests (data not shown).

Plants with no stress showed no difference in photosynthesis between treated and control, a response similar to the one found on non-irrigated plants, however at a much lower level, when comparing the non-stressed with the non-irrigated. Plants infected with the pathogen but treated with the beneficial bacterium showed a photosynthesis level higher than untreated control or plants subjected to drought stress but lower than the non-stressed plants. Untreated and infected plants had photosynthesis close to zero, a result worse than drought stressed plants (no irrigation) for eight days.

When plants were irrigated and photosynthesis assessed 24h afterwards, a higher level of photosynthesis was found in plants treated with the beneficial bacterium compared to untreated control and the same response was found in plants previously subjected to drought stress.

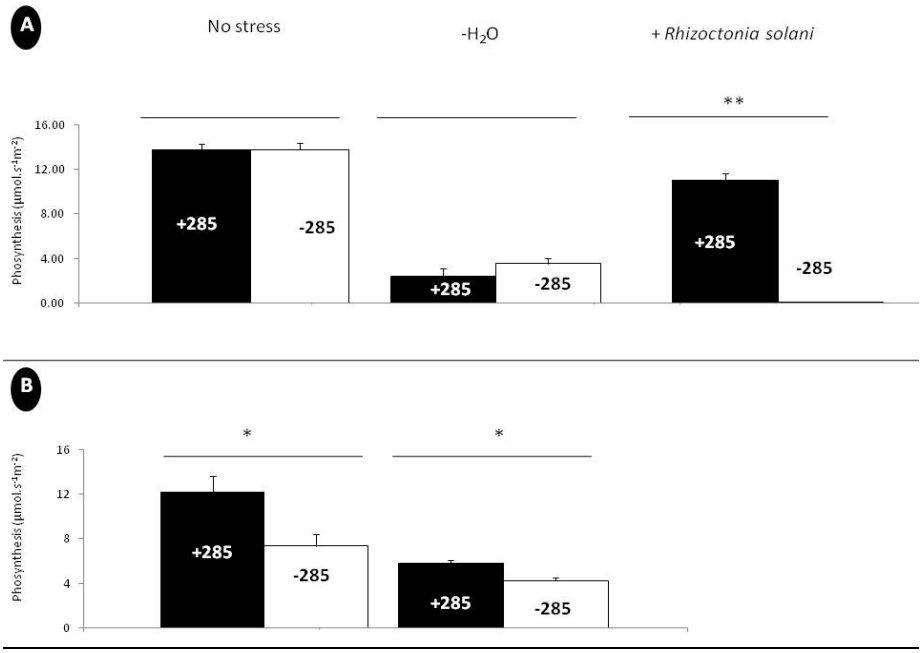

FIGURE 8 Photosynthesis was measured for plants originated from seeds treated with *Bacillus subtilis* UFLA285 (+285) or untreated plants (-285) at 17 days after sowing and 8 days after inoculation (A). The treatment of the rhizobacterium induce na increase in photosynthesis at 19 days after sowing after an 8-day water stress and photosynthesis measured 24h after irrigation (B)

In order to confirm the photosynthesis measurements, plants were harvested nearly one week after the last measurement and the shoot dry weight was recorded (Table 4).

Plants subjected to no stress were similar ($p = 0.42$), however those subjected to a eight-day water stress and then regularly watered until sampling were higher ($p = 0.04$) by 36% on rhizobacterium-treated over control and the treatment also assured a higher ($p = 0.008$) dry matter by 90% on plants inoculated with *R. solani*.

For each treatment level (treated or not with the rhizobacterium), differences were also observed. For rhizobacterium-treated plants, those inoculated or not were similar and both were higher than the water stressed plants. For control plants, both inoculated and water stressed plants were similar and both were lower than the non-inoculated and watered plants (no stress).

TABLE 4 Shoot dry weight (g/seedling) of cotton plants at 25 days after sowing (DAS). Either treated (+285) or untreated (-285) with *Bacillus subtilis* UFLA285 and regularly watered and non-inoculated (no stress), subjected to an eight-day water stress and then regularly irrigated (-H_2O) or inoculated with *Rhizoctonia solani* AG4 strain 1 (+*Rizoctonia solani*) at 9 DAS.

Stress / Treatment	No stress	-H_2O	+*Rhizoctonia solani*
+285	0.99 a A	0.60 aB	0.93 aA
-285	0.91 a A	0.44 b B	0.49 b B

Data measured in grams per plant and averaged for four of them. Means followed by the same lower case letters in the columns and capital letters in the rows are similar according to Tukey's test ($p \leq 0.05$)

6 DISCUSSION

In a previous work, Medeiros et al. (2008) screened over 300 endospore-forming bacterial strains for the control of two important seed-borne diseases of cotton: bacterial blight (*Xanthomonas axonopodis* pv. *malvacearum*) and damping-off/ramulose (*Colletotrichum gossypii* var. *cephalosporioides*) aiming at a broad sprectrum activity and obtained *Bacillus subtilis* UFLA285. The rhizobacterium reduced post-emergence damping-off in the field and reduced seed-borne associated fungi. The broad spectrum nature has proved to be operative, since in the present work, the rhizobacterium-seed treatment protected plants against damping-off caused by *Rhizoctonia solani* AG4. The control was effective only when plants were inoculated 9 days after sowing which was the time necessary for the onset of benzothiadiazole-mediated systemic acquired resistance against the same disease (Jabaji-Hare & Neate, 2001).

The requirement of a time for the plant response to the pathogen was an indication of induced systemic resistance as shown by the up-regulation of genes putatively associated to defense responses. All genes had been selected from Dowd et al. (2004) who studied genes associated to cotton seedlings response to *Fusarium oxysporum* f.sp. *vasinfectum* a vascular wilt causing pathogen. Those gene expression were thus likely to be up-regulated on cotton infected seedlings compared to healthy ones and the observation of genes up-regulated on rhizobacterium-treated over untreated and infected seedlings represent a synergistic action of the symbiont on the already existing plant defense potential. Although both stem and roots were infected by the pathogen, the observed symptoms were more pronounced on stems which might explain the presence on this plant part of a consistent up-regulation of ethylene

inducible protein, marker gene of the jasmonate-mediated inducible systemic resistance pathway as well as peroxidase at the last time point.

On earlier sampled time points, genes were conversely down-regulated, ethylene inducible protein on roots (10 DAS), peroxidase on root (10-11 DAS) and stems (10DAS). A similar finding was found by Wang et al. (2005) studying tomato genes activated by the presence of *Pseudomonas fluorescens* FPT9601-T5. They observed a down-regulation of jasmonate/ethylene defense pathway related genes, lipoxygenase (At1g72520), ethylene responsive factors (At1g74930, At2g22200, At2g44840, At4g34410 and At5g47220) as well as a phenylpropanoid pathway-related one, cinnamoylCoA reductase (At5g14700). The authors postulated this phenomenon as a strategy for the rhizobacterium colonization and that the use of the biocontrol agent represents a partially effective disease control strategy at that sampled time point.

However, in the presently studied system, as fungal infection took place, gene expression switched from a down-regulation to unchanged and then to up-regulation pattern to face the disease, since at the last time point (13 days after sowing DAS), both root and stem showed an up-regulation of peroxidase, ethylene inducible protein and on roots an up-regulation of chitinase, this was the time point where symptoms were visible in all plants (Fig 1C). At that time, microarray analysis was performed and the genes mentioned as down-regulated by Wang et al. (2005) were all up-regulated (Table 3).

The microarray analysis revealed a total of 246 genes with changed regulation. They were associated to categories according to the putative function and grouped in primary or secondary metabolism and the link between them has been addressed in the metabolic pathway (Fig 4) likely to be operative, showing the primary metabolism directed to energy as well as the anapleurotic branches from oxaloacetate and phosphoenolpyruvate leading to the secondary metabolism.

The primary role of the citric acid/electron transport chain is the energy generation which is used for active transport and anabolism. The synthesis of sterol lipids is likely to occur through ketoacylCoA. Those macromolecules are used for the synthesis of plant hormones (brassinesteroid and jasmonate) whose receptors have been up-regulated (brassinosteroid-regulated protein and lipoxygenase/allene oxyde synthase) in our study (Table 1) as well as cell and organelle membranes (Demel & De Kruyff, 1976).

From oxaloacetate, starts another anapleurotic branch involved in the synthesis of arginin (asparagines tRNA ligase was up-regulated) and the product is converted to glutamic acid and then proline. The proline accumulated in cotton plants infected with *R. solani* and this increase was 30% higher on rhizobacterium treated plants (Figure 6). The aminoacid is reported as accumulating in drought stressed plants (Bates, 1973) and for the first time it has also been related to fungal infection. Its build up has a dual role: evidences suggest not only the osmorregulation but also enzyme stabilization, specially RUBISCO (De La Rosa et al., 1995; Solomon et al., 1994). The enzyme stability under stress is also assured by other gene antioxidants (thioredoxin) or metabolic pathways (phenylpropanoid branch leading to antocyanins) found to be up-regulated.

The defense responses followed a typical induced systemic resistance pathway with the up-regulation of jasmonate (lipoxygenase, allene oxyde synthase) as well as ethylene (ethylene receptor, ethylene binding protein) signaling molecules and the down-stream defense responses pathogenesis-related protein with reported activity against Eumycota or "true" fungi (thaumatin-like protein, endo-beta-acetylglucosaminidase) (Thompson et al., 2006; Mamarabadi et al., 2009) and Oomycota (uncharacterized resistance protein similar to a *Quercus* sp one) (Ana Coelho, unpublished data). The broad range of activity against other pathogens has already been proven (Ferro et al.,

2008) but the other PRs have not yet been proven to be the mechanism reported with this activity. The tested rhizobacterium has shown activity against bacterial blight, a bacterial pathogen, but none of the PRs has yet been previously reported.

The phenylpropanoid pathway was also activated on treated plants, with several branches found as operative: the undiferenciated initial step (phenylalanine ammonia lyase), phytoallexins (2-hydroxyisoflavone reductase), catechins/anthocyanins (dihydroflavonol 4 reductase), phenolics/lignin (caffeic acid O-methyltransferase, cinnamoyl CoA reductase) (Zabala et al., 2006). Catechins as well as tannins, its oxidation product, are the main polyphenols in cotton and produced in high amounts in response to *Rhizocotonia solani* infection (Kirkpatrick & Rothworth, 2001). The tannins inhibit fungal polygalacturonases, responsible for the tissue maceration (Kirkpatrick & Rothworth, 2001). The over-expression of a pathway leading to the synthesis of those compounds by the rhizobacterium treatment demonstrates its role in boosting up the natural plant responses to the pathogen.

The lignin, also a product of the phenylpropanoid pathway, represents a physical barrier for the fungal infection which is complemented by other cell wall reinforcement strategies and a down-regulation of those of cell wall loosening (Table 3). A lipid transfer protein as well as a callose synthase genes, coding respectively for cuticle formation and the callose deposition were found to be up-regulated and they are involved in preventing fungal penetration. A down-regulation of xyloglucan endoglycosyl transferase, which acts on the cell wall loosening for hemicelulose deposition on cell expansion, helps reducing the natural openings for fungal invasion.

Another important player in secondary cell wall reinforcement is cellulose. In cotton , the cellulose synthesis is a crucial step in fiber formation, its synthesis occurs from sucrose through the action of sucrose phosphate

synthase resulting in fructose and UDP glucose, the last is polymerized by cellulose synthase to cellulose (Babb & Heigler, 2001). The sucrose phosphate synthase was found to be up-regulated while cellulose synthase was down-regulated (Table 2) suggesting for a build up in UDP glucose in the infected plant which might be justified by the higher energy requirement or accumulation of the hexose in vacuoles in order to maintain the cell turgor under osmotic stress.

To make the information go through it would be expected to find an up-regulation of signal transduction and transcription factors. The first is initial steps on induced systemic resistance since transmembranic domain that perceives the elicitor (Avr9/Cf9 rapidly elicited protein, serine threonine and leurice rich repeat kinases) and has an internal domain that phosphorilate proteins in kinase, cascades, reaching the nucleus for the transcription (Buchanan, 2000). One of the recent *Bacillus subtilis*-based induced systemic reistance is the reversible disturbance in the cytoplasmic membrane by bacterial-born surfactant and the plant response is the activation of membrane-anchored signal transduction proteins leading to disease resistance induction (Jourdan et al., 2009).

Interestingly, 20% of kinases found to be up-regulated in the system were calcium-dependent (Table 3). This mineral also takes part as cofactor in a variety of biological process such as collose synthesis (Buchanan et al., 2000), an this gene was also up-regulated. denoting the important role of the mineral in the disease resistance induction system presently studied.

Also found as important as the calcium dependent at the same rate was the hormone-realted signal transducers (Table 3). Plant hormones were also the the most important group (22% of total) of the transcription factor category (Table 3). Among the hormone receptors, the ethylene ones were the most frequent. Although, this molecule was not quantified, this hormone is reported

as part of the induced systemic resistance signaling (Pieterse et al., 2007), along with jasmanate, which was also discussed as likely to be present in the studied system.

Another transcription factor has been reported as insect response (wound-induced leucine zipper zinc finger) (Table 3), which shares the same jasmonate/ethylene signaling pathway and is important disease resistance molecular markers in cacao (Barrone et al., 2004). No pathogenesis-related protein associated to insect control such as proteinase inhibitor was found to be up-reagulated and this may be due to the fact that the plant responses fine tunes to pathogen resistance instead of broad spectrum activity.

The necrotrofic infection such as the one produced by *R. solani*, generates reactive oxygen species (ROS), which are removed by scavengers found to be up-regulated (peroxidase and glutathione-S-transferase) (Able, 2003), a similar response on drought stressed plants (Ramanjulu & Bartes, 2002).

The pool of toxic metabolites either produced by the fungus or the plant-pathogen interaction is restricted to ROS. A carbohydrate containing glucose, mannose, N-acetylgalactosamine and N-acetylglucosamine has been found as a virulence factor in *R. solani* sheath blight in rice and the toxin has also been found in cotton-infecting isolates (Vidhyasekaran et al., 1997). To either eliminate the toxin from inside the cell or degrade it to non-toxic compounds, the plant potentially produces specialized proteins with exocytosis (MATE efflux family protein) or degradation (cytochrome P450 and endo-beta-Nacetylglucosaminidase) (Eckardt, 2001; Ralston et al., 2001; Mamarabadi et al., 2009).

Not surprisingly, photosynthesis was much higher on rhizobacterium-treated and subsequently infected plants (Figure 8A) even though severe symptoms were seen even on treated plants. At 19 days after sowing, untreated

plants died, but treated plants remained alive and up-right with turgid leaves that were as photosynthetically active as the non-stressed untreated control at the same age (data not shown). At 13 days after sowing, when proline analysis was performed, no difference between treated and untreateed, both subjected to drought stress, was observed, but 6 days later, photosynthesis was higher for both treated and control, which can be related to a direct growth promotion effect, proline-assured RUBISCO integrity or both strategies. The hypothesis will be validated in future experiments.

Photosynthesis activity is highly dependent on water availability and its cell-to-cell redistribution to reach phosynthetic tissues (Abdeeva et al., 2008). The water availability, as already discussed, was assured by the accumulation of osmolites (proline and possibly glucose) while the redistribution was assured by aquaporins. Under drought stress or symbiont association, this gene is up-regulated in plants (Tyerman et al., 2002) and in both rhizobacterium association and drought stress treatments without inoculation we observed an increase in the expression of this gene (Figure 7).

In the studied plant pathogen interaction, followed by tissue necrosis, even without complete tissue girdling, on untreated and inoculated plants, a wilting symptom was observed similar to the that caused by a water stress or *Fusarium oxysporum* f.sp. *vasinfectum* infection. Although *Rhizoctonia solani* has not been reported as being a xylem colonizer (Kirkpatatrick & Rothrock, 2001) from the initial infection, cushions formed on the stem epidermis, the mycelium reached and damage the tracheary elements reducing the water conductivity and thus, with normal water transpiration (data not presented), a negative water balance occurs leading the plant to an irreversible wilting. On treated plants, in spite of the brownish necrotic lesions (Fig 1B) no wilting symptom was observed possibly due to a lower extent of the pathogen internal tissue colonization from cell wall reinforcement (callose and lignin deposition)

as shown for the binucleate *Rhizoctonia* mediated biological control of damping-off (Cardoso & Echandi, 1987). Surprisingly, rhizobacterium treated and infected plants did not improve the aquaporin level, as was observed for rhizobacterium-treated submitted to no stress. A down-regulation of aquaporin under a lack of water situation is not an exception to the rule (Kirch et al., 2000; Mariaux et al., 1998; Sarda et al., 1999) and this is a plant strategy to avoid losing water in this specific case, the down- regulation would be a strategy for the rhizobacterium treated plant to avoid supplying water to infected tissues. The aquaporin regulation may yet be tissue specific or even organelle specific. Kirch et al. (2000) postulated that while vacuole-rich cells under a lack of water show a down-regulation of aquaporin, this gene may be up-regulated on endosome trafficking of roots, increasing its water uptake.

The presented article postulated evidence of similarities between rhizobacterium-mediated damping-off control and drought stress protection from net photosynthesis measurements, shoot dry weight, proline accumulation, aquaporin regulation and ROS scavendger production, which, combined with classical jasmonate/ethylene mediated induced systemic resistance, has resulted in efficient disease control. However, the wide use of induction of systemic resistance-mediated biological control is hampered by a metabolic cost constraint (Heil, 2001). An up-regulation of genes associated with respiration (cytochrome b5 and 4-phosphopantothenoylcysteine synthetase) as well as a down-regulation of a photosynthesis-related gene (light harvesting complex) (Table 3) would be an argument for a detrimental performance in the primary plant metabolism. However no difference in net photosynthesis measurements between treated and control in any of the stress situations (no stress, pathogen inoculation or no irrigation) or when present, the difference was in favor of the rhizobacterium-treated (Figure 8). Furthermore no differences in plant dry weight between treated and control (Table 4) suggested that the metabolic cost

involved in the rhizobacterium-mediated changes were not likely to represent a detrimental effect on the overall plant development. The rhizobacterium treatment assured that inoculated plants had a similar plant dry matter than non-stressed/non-inoculated plants either treated or not but higher than untreated inoculated plants. The rhizobacterium could not reestablish the normal plant growth on water-stressed plants but the performance was better than the untreated ones, which suggest a plant protection not only against the pathogen infection but also against water stress.

The multiple features of the studied *Bacillus subtilis* strains, i.e. protection against biotic and abiotic stresses, combined to the on-going formulation experiments will provide cotton growers with an extra tool to improve the crop performance.

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GENERAL CONCLUSIONS

- Two rhizobacterial strains: *Bacillus subtilis* UFLA285 and *Paenibacillus lentimorbus* MEN2 reduced both damping-off caused by *Colletotrichum gossypii* var. *cephalosporioides* and bacterial blight caused by *Xanthomonas axonopodis* pv. *malvacearum*
- The strains assured germination similar to the non-inoculated control both under controlled conditions (greenhouse) and in the field when they were used in combination in two consecutive growing seasons.
- They also controlled post-emergence damping-off in the first year of the trial, reduced the population of seed-associated fungi, increased the bacterial one.
- Shoot dry weight for seedlings originated from seeds treated with each antagonist under Cgc inoculum pressure was similar to the untreated and non-inoculated control and in the field no difference was found between treated with the mixture, UFLA285, fungicide or water control and MEN2 had a detrimental effect on plant growth compared to the water control
- UFLA285 was also effective in the control of damping-off caused by *Rhizoctonia solani* when plants were inoculated 9 days after sowing.
- The rhizobacterium induced the expression of ethylene inducible protein and peroxidase in both stem and roots, especially four days after inoculation (13 days after sowing).
- A total of 246 genes had changed regulation, among which typical jasmonate/ethylene-mediated induction of resistance and the phenylpropanoid pathway-related.
- Responses peculiar to drought tolerance: proline synthesis and accumulation as well as aquaporin regulation were operative.

- Plants originated from rhizobacterium-treated seeds displayed higher photosynthesis than the water treated control and showed a more rapid reestablishment of normal photosynthetic rate once the water status is reestablished in the plant.
- The dual role of simultaneously facing biotic and abiotic stresses has been reported and shed light on a possible novel disease control mechanism.