MURTALA ISYAKU

PARASITISM OF *Ramulariopsis pseudoglycines* IN COTTON GENOTYPES WITH DIFFERENT LEVELS OF RESISTANCE.

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Dissertation presented to Graduate Program in Agronomy/Phytopathology, area of concentration in Phytopathology, Federal University of Lavras, as part of the requirements to obtain the Master Degree M.sc

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PARASITISMO DE *RAMULARIOPSIS PSEUDOGLYCINES* EM GENÓTIPOS DE ALGODÃO COM DIFERENTES NÍVEIS DE RESISTÊNCIA

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RESUMO

A mancha foliar de Ramularia (MFR), causada por *Ramulariopsis pseudoglycines*, é uma das doenças que acometem o algodoeiro no Brasil. A doença é amplamente disseminada na região produtora de algodão brasileira. O objetivo deste estudo foi identificar o estádio crítico na folha do algodoeiro em que a doença se desenvolve pelo uso de diferentes técnicas de microscopia. A caracterização histopatológica comparativa está entre os estudos realizados na avaliação de cultivares resistentes a doenças. Três cultivares de algodão com diferentes níveis de resistência, IMA 5801B2RF (altamente resistente) (I), DP1746 B2RF (alta susceptibilidade) (D), TMG44 B2RF (moderadamente resistente) (T) e dois isolados de *R. pseudoglicynes* (FAL F3 (A) e FAL FICC (B)) foram utilizados neste estudo. Técnicas de microscopia (Microscopia de Luz) (ML) e Microscopia Eletrônica de Varredura (MEV) foram utilizadas para caracterizar a interação de *R. pseudoglycines* com tecidos foliares de genótipos de algodoeiro com diferentes níveis de resistência a MFR. Usando delineamento de blocos completamente casualizados, um total de 60 vasos de plástico foram usados no cultivo das três cultivares de algodão. Para o teste de severidade foram utilizados 30 (trinta) vasos plásticos com plantas de algodão das três cultivares. Os outros 30 foram usados para as análises microscópicas. Após trinta dias de germinação das plantas em casa de vegetação procedu-se a inoculação. As plantas foram então transferida para câmara de crescimento e mantidas no escuro, sob temperatura de 25 a 30^{o}C e umidade relativa de 100% por 24 horas sendo depois colocadas em regime de 12 horas de luz. Com os dados da severidade foi análise para determinada a Área Abaixo da Curva de Progresso da Doença (AACPD), utilizando o programa R, para cada cultivar e isolado. As amostras para análises microscópicas foram coletadas às 12, 24, 48, 72 e 96 horas após a inoculação (hai). A análise histopatológica mostrou maior acúmulo de compostos fenólicos na cultivar IMA 5801 (altamente resistente), seguida das cultivares TMG44 (moderadamente resistente) e DP1746 (alta suscetibilidade). Na análise em MEV, observou-se alta taxa de germinação e infecção no início do processo infeccioso da cultivar DP1746 (alta suscetibilidade), enquanto para as demais cultivares com maiores níveis de resistência, alta taxa de conídios não germinados foi observada em fases avançadas do processo infeccioso. A maior AACPD foi observada na cultivar DP1746 inoculada com o isolado B, e as demais cultivares, tanto inoculadas com o isolado A, quanto B não diferiram estatisticamente.

Palavras-chave: Mancha foliar de Ramularia. Algodoeiro. *Ramulariopsis pseudoglycines*. Resistência a doenças. estudos microscópicos.

ABSTRACT

Ramularia Leaf Spot (RLS), caused by *Ramulariopsis pseudoglycines*, is one of the main diseases that affect cotton in Brazil. The disease is widely spread in Brazilian cotton producing region. The aim of this study was to identify critical stage on the leaf of cotton in which disease develops by the use of different microscopy techniques. The comparative histopathological characterization is among the study carried out in the evaluation of the disease resistant cultivars. Three cotton cultivars with different levels of resistance: IMA 5801 B2RF (highly resistant) (I), DP1746 B2RF (high susceptibility) (D), TMG44 B2RF (moderately resistant) (T) and two isolates of *R. pseudoglycines* (FAL F3 (A) and FAL FICC (B)) were used in this study. Microscopy techniques: Light Microscopy (LM) and Scanning Electron Microscopy (SEM) were used to characterize the interaction of *R. pseudoglycines* with leaf tissues of cotton genotypes with different levels of resistance to RLS. Using completely randomized block design, a total of 60 plastic pots were used in growing three cotton cultivars. Thirty (30) plastic pots of cotton seed containing 10 plants of each evaluated cultivars were used for severity test. The other 10 plant for each evaluated cultivars were used in determining the appearance of RLS using microscopy in the laboratory, totaling 30 plants. Plants with thirty days after germination, were kept in greenhouse, and inoculated with the isolates. The inoculated plants were transferred to dark growth chamber, for 24h, in order to carry out the experiment under the temperature range of 25 to 30ºC, 12 hrs of light and 100% of relative humidity. Severity data were analyzed using the R program by means of the Area Under the Disease Progress Curve (AUDPC). Samples for microscopic analysis were collected at 12, 24, 48, 72 and 96 hours after inoculation (hai). Histopathological analysis showed higher phenolic compounds accumulation in cultivar IMA 5801 B2RF (highly resistant), followed by cultivars TMG44 B2RF (moderately resistant) and DP1746 B2RF (high susceptibility). In the SEM analysis, a high rate of germination and infection was observed at the beginning of the infectious process of the DP1746 B2RF cultivar (high susceptibility), while for the other cultivars with higher levels of resistance, a high rate of non-germinated conidia was observed in advanced stages of the infectious process. The highest AUDPC was observed in the DP1746 B2RF cultivar inoculated with isolate B (high susceptibility), and the other cultivars, both inoculated with isolate A and B, did not differ statistically.

Keywords: Ramularia Leaf Spot. Cotton**.** *Ramulariopsis pseudoglycines.* Plant disease Resistance. Microscopy

SUMMARY

1. INTRODUCTION

Ramularia leaf spot is one of the main diseases of cotton (*Gossypium hirsutum* L.) in Brazil. In the recent study by Silva et al., (2021), 252 out of 267 isolates of *Ramulariopsis spp.* that were evaluated in cotton plants from six Brazilian states and the Federal District were identified as *R. pseudoglycines.* This indicates that the species is the most widespread causal agent of cotton RLS in Brazilian producing regions today. It was observed that isolates of *R. gossypii* were restricted to small and isolated farms located in the Federal District and Paraiba state, while isolates of *R. pseudoglycines* were obtained from all sampled sites, all from extensive farms in the Brazilian Cerrado, this being the most representative species in the pathosystem (SILVA et al., 2021).

The management of the disease is challenging, with often large number of fungicide applications during the cotton season, which minimize production and might results in quality losses (BETTIOL; DA SILVA; SUASSUNA, 2019; TORMEN; BLUM, 2019). This large number of fungicides applications has resulted in selection pressure and prevalence of isolates of *R. pseudoglycines* resistant to specific site in evaluated populations (MATHIONI et al., 2021). Anti-resistance strategies associated with efforts to develop genetic, biological and cultural control measures may result in a more rational use of fungicides (SILVA et al., 2021). Among these measures, use of genetic control RLS-resistant cotton genotypes is one of the most important way that reduce the use of fungicides, environmental contamination, selection pressure on populations of fungi resistant and production costs.

The construction of RLS management strategies in cotton should be based on knowledge of the biology of *Ramulariopsis pseudoglycines*, plant-pathogen interaction and pathosystem-associated epidemiology. Literature reports have demonstrated the influence of each vertex of the disease triangle for this pathosystem, where the infectious process is influenced according to different *Ramulariopsis* isolates, cotton genotypes and environmental conditions.

The presence of initial inoculum such as conidia on volunteer cotton plants or ascospores on decomposing cotton leaves can anticipate the first symptoms to 20 days after crop emergence. These begin as pale green to yellowish-green lesions on the adaxial leaf surface, where the white powdery sporulation of the fungus begins. Lesions about 4 mm wide are delimited by the veins, giving them an irregular and angular contour, which expand to chlorosis and tissue necrosis. In most cultivars, the pathogen behaves as a biotrophic, producing

conidia on living leaf tissues, on both abaxial and adaxial faces (BETTIOL; DA SILVA; SUASSUNA, 2019).

Maximum conidia germination on cotton leaves was observed 12h after inoculation, usually with one or two germ tubes. These grow at random and penetrate without the formation of appressoria through open stomata (CURVÊLO et al., 2013). Night duration(12h) and leaf wetness and daytime drying favor the penetration of the pathogen (RATHAIAH, 1977). Curvelo, (2013) reported an ideal temperature range between 25 to 30 °C for spore germination and germ tube elongation. After penetration, the mycelium was observed to colonize intra and inter leaf mesophyll. As a consequence, the cells collapse at 16 days after inoculation. Early sporulation without penetration was already observed four days after inoculation in susceptible cultivar BRS286 in a growth chamber with 100% humidity. However, sporulation after penetration and colonization occurs 12 days after inoculation (CURVÊLO et al., 2013). Conidiophores emerge in clusters through the stomata and sporulation occurs on the adaxial and abaxial faces of the leaves. When evaluating the effect of temperature on sporulation of *R. pseudoglycines in vitro,* Galbieri et al., (2015) found that fungal sporulation was minimal at 12 °C and no sporulation occurred at 33 °C. It was also observed that the optimum temperature of 17 °C for sporulation of isolates from São Paulo and 23 °C for isolates from Goiás and Mato Grosso states. The evaluation of the aggressiveness of isolates from different geographic regions of Brazil evaluated on cotton leaves 20 days after inoculation showed that the isolates differed in spore production per leaf area (GALBIERI et al., 2015). Several studies have demonstrated the variation in resistance and susceptibility reactions of cotton genotypes according to the *Ramulariopsis* isolate (ALVES et al., 2013; PEZENTI et al., 2013; GALBIERI et al., 2015).

Among the studies carried out in the evaluation of disease resistant cultivars, the comparative histopathological characterization is very useful for the elucidation of the plant-pathogen interaction, making it possible to detect critical stage for the pathogenesis, details of the pathogen's infectious process, plant defense responses and components of structural or constitutive defense of the plant. This information is of high value for the selection of lines in genetic improvement programs. Several tools are used, from techniques that require less resources, such as light microscopy (LM) and histochemistry, to those that require very expensive reagents and equipment, in addition to more sophisticated techniques, such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), fluorescence and laser confocal microscopy (LCM). An important study carried out to characterize the

interaction of the *Plasmopara viticola* pathosystem in resistant and susceptible vine genotypes was published in the Horticulture Research section of Nature Magazine (YIN et al., 2017). In this work, several aspects of the cell biology of the interaction were understood using LM, SEM, TEM and LCM.

The components related to plant defense that are important in the resistance of cotton to RLS for each pathogen, as well as relative measure quantifications that will present statistically significant differences. In addition, the occurrence of different evidence in anatomical characteristics was verified. However, practically little was known about the histopathological and ultrastructural aspects of the *Ramulariopsis* and cotton interaction. In order to improve methods for evaluating the resistance of cotton cultivars to ramularia leaf spot, it is proposed to use several microscopic techniques (LM, SEM and LCM) to characterize the interaction of *Ramulariopsis pseudoglycines* with leaf tissues of cotton genotypes with different levels of resistance to RLS.

The main objective of the study is to identify critical stage for pathogenesis. More specifically: I. To evaluate the different level of cotton genotype resistance to the pathogen. II. To know details of the pathogens' infectious process using different types of microscopes techniques such as LM, SEM and LCM. III. To know plant defense responses. IV. To know structural or constitutive defense of the plant. This information will be of high value for future studies on the control of ramularia leaf spot on cotton and for the selection of lines in genetic improvement programs.

2. BIBLIOGRAPHIC REVIEW

2.1 Cotton Plant

The genus *Gossypium* L. (collectively termed cotton) comprises about 50 species distributed globally. Different ancient human cultures at the different continents, independently, have domesticated four cotton species - two allopolyploids from the Americas, *G. hirsutum L.* and *G. barbadense L.*, and two diploids from Africa-Asia, *G. arboreum L*. and *G. herbaceum L.* (WENDEL; GROVER, 2015). *Gossypium hirsutum L*. latifolium Hutch, commonly known as upland cotton, is an important commodity in the agricultural economy of the world and accounts for over 90% of world production. *Gossypium barbadense*, also known as Pima, Sea Island, Egyptian, or extra-long staple, represents approximately 5% of world fiber production (ADAMS, 2015).

Since the 1990s, upland cotton growing area has increased substantially in the Brazilian Cerrado (savannah), mainly due to breeding efforts for developing locally adapted high yielding cultivars, as well for improving agronomic practices (MORELLO et al. 2015; SILVA NETO et al. 2016; BARROSO et al., 2017; SUASSUNA et al. 2018). Cotton crop was introduced into the Cerrado to integrate cropping systems that include soybean and corn. Currently, the largest cotton-producing area in Brazil is located at the Cerrado where acreage is extensive and dominated by a few cultivars (FERREIRA FILHO; ALVES, 2007). Such scenario exacerbates the impact of an emerging disease that reaches severe epidemic levels quite often such as Ramularia leaf spot (RLS).

2.2 Ramularia Leaf Spot (RLS)

The first report of RLS on cotton was in Auburn, AL, United States (Atkinson 1890). In Brazil, RLS for long was considered of secondary importance (CIA 1977; CIA AND SALGADO, 1997) but emerged as the most important cotton disease after the large expansion of cotton production in the Cerrado where new and susceptible cotton cultivars have been grown mostly under monoculture (SUASSUNA AND COUTINHO, 2014). Extend period of high air humidity and daytime temperature ranging from 25 to 30^{o} C, which are favorable for RLS epidemics (RATHAIAH, 1977) are prevalent during the growing season in the Cerrado. The

occurrence of damaging epidemics has led to intensive use of fungicide sprays, which increases production costs as well as the risk of fungicide resistance

2.2.1 Economic Importance of the Disease

Currently, up to eight sprays of fungicide are required every season in an area of more than one million hectares, which increases production costs to U$ 160 million annually. The increasing importance and the economic impact of RLS in Brazil have been highlighted in previous studies (MEHTA AND MENTEN 2006; AQUINO et al. 2008; BARROS et al. 2008; SILVA et al., 2017). Reduction in fiber yield due to RLS ranges from 20 to 60% (CAUQUIL and SÉMENT 1973; SHIVANKAR and WANGIKAR 1992; AQUINO et al., 2008) depending on cotton genotypes, planting season and geographical location. Although effective control can only be achieved by applying fungicides (SUASSUNA and COUTINHO, 2015), cotton cultivars with high resistance level to RLS have been developed and released recently and are now part of disease management program together with cultural, biological and other control methods.

2.2.2 Disease Symptoms

The first symptoms of RLS generally appear at the lower canopy, usually after the first squares (flower bud), between 4 and 8 weeks after planting, depending on the cultivar and environmental conditions. However, in fields with volunteer (self-sown) cotton plants and/or cotton stalk regrowth, primary inoculum is available earlier and first symptoms can appear as early as 20 to 25 days after crop emergence. Such scenario may lead to explosive epidemics towards the end of the season. Initial symptoms appear as light green to yellow-green lesions on the upper leaf surface from which the fungus develops white powdery sporulation (BELL, 1981). Lesions (3–4 mm in width) are delimited by the veinlets, giving them an irregular, angular outline and for the most cotton cultivars, the pathogen behaves like a biotrophic, producing spores in green live plant tissue. Afterwards, the lesion expands causing chlorosis and leaf necrosis. Under high humidity conditions, the sporulation may occur on both sides of the leaves and the disease is easily found on the middle and further on top of the plant. The disease can accelerate defoliation, which contribute to reduce both the yield and the fiber quality. Early defoliation can be complete in susceptible cultivars.

2.2.3 Disease Causal Agent

Ramulariopsis pseudoglycines (Synonyms: *Ramularia areola* Atk., *Ramulariopsis gossypii* (Speg.) U. Braun, *Ramularia gossypii* (Speg.) Ciferi, Cercosporella gossypii Speg.), is the anamorphic form of *Mycosphaerella areola* Ehrlich & Wolf. The fungus develops in three distinct stages during its life cycle. The conidial stage occurs in living tissues, while the leaves remain attached to the plant, and for a short period after their abscission. Conidia can remain viable for 35 days under natural field condition and, under refrigerated condition, remained viable up to 115 days (GAVIYAPPANAVAR, 2012). In dehydrated cotton leaves with silica gel kept under storage conditions, the conidia viability (germination) was observed for more than 200 days. The spermogonia stage occurs after leaf fall and is followed by the ascogenous stage, which develops in partially decayed leaves (EHRLICH and WOLF, 1932). This fungus is reported as pathogenic only in species of the genus Gossypium.

The anamorph phase of the fungus was described in 1883 from cotton material collected in Paraguay and named *Cercosporella gossypii* Speg. (SPEGAZZINI 1886). Subsequently the disease was diagnosed in 1890 in the state of Alabama, United States by Atkinson, who described its etiological agent as *Ramularia areola* G.F. Atk. In 1993, the species was reclassified to *Ramulariopsis gossypii* (Speg.) U. Braun (BRAUN, 1993) and recently, an extensive polyphase study of *Ramularia sensu lato* indicated that the causal agent of the RLS is better classified as *Ramulariopsis pseudoglycines* (VIDEIRA et al., 2016). However, as the common name of the disease is long established, we will continue to treat it by RLS.

From the anamorph, it is possible to observe conidiophores emerging from pseudoparenchymatous tissue through the stomata in loose fascicles, (sub) hyaline, up to 70 μm in length, sympodially proliferating, with 2–6 conspicuous scars. Stroma is not present. Conidia arising in short chains, hyaline, thin-wall, punctate, fusiform, 1 to 3 septate, mostly 20–30 × 4 μm (HOOG, 1992). The teleomorphic stage was reported only in USA (EHRLICH and WOLF, 1932), South Africa (GOWS et al., 2001) and Brazil (MEHTA et al., 2016), Gows et al. (2001) observed the presence of dark brown pseudothecia, 80–90 μm in diameter, and slightly beaked, on decayed leaves and prematurely opened bolls. Asci were hyaline, fusiform, 50–60 (72) × 6–8 μm and contained eight ascospores per ascus.

Ascospores were 1-septate, hyaline, 12–15 × 2–3 μm. Similarly, Mehta et al. (2016) identified ascoma of *Mycosphaerella sp.* were 40–60 μm in diameter. They contained several

hyalines, bitunicate asci with a distinct foot cell. The size of the asci varied between 54.1 and 67.8 μm in length. Each ascus contained one septate eight ascospores, showing constriction at the septum. The ascospore were hyaline, measured 6.6 × 18.2 μm and germinated by both cells, similar descriptions of the *Mycosphaerella areola* fruiting bodies reported by Ehrlich and Wolf (1932).

2.2.4 Disease Epidemiology

The RLS pathogen is able to survive on cotton volunteer plants and on cotton stalk regrowth, generally when unsuccessful cotton stalk destruction occurs. These survived plants are found in the field before and during sowing of the new crop season. Growing cotton at the same field for several years, or nearby fields with volunteers and stalk regrowth, may lead to build up of primary inoculum that will be available early for the new crop at the beginning of the season.

3. MATERIAL AND METHODS

3.1 Plant Material and Microorganisms produce

Three cotton cultivars with different levels of resistance to RLS were used in this study: DP1746 B2RF (high susceptibility) (D); TMG44 BR2RF (moderately resistant) (T) and IMA5801 BR2RF (highly resistant) (I) (PEDROSA et al., 2020).

The cotton plants were grown in plastic pots containing 1.5 kg of substrate composed of a soil mixture of the Agricultural soil Red Yellow class, clayey texture, sand and farmyard manure in the proportion of 3:1:1. Soil acidity was corrected with 2 g of dolomitic limestone kg $^{-1}$ of substrate. Simple superphosphate, ammonium sulfate, potassium chloride and magnesium sulfate (10; 0.75; 0.25 and 0.5 g kg $^{-1}$ of substrate, respectively) were added five days before sowing (CURVELO et al., 2010). The plants were kept in a greenhouse and watered daily.

Two cultures of isolates of *R. pseudoglycines* (FAL F3 (A) and FAL FICC (B)) were used in this study. Mycelium disks colonies grown in Potato-Dextrose-Agar (PDA) medium (Figure 1A) were transferred to crucibles, and macerated with the sterilized distilled water. 200 μL volumes of the macerate was transferred to Petri dishes containing V8-Agar culture medium

(V8 Vegetable Juice, Campbell's ®), then spread over the surface of the medium. Incubation was carried out at 25 ºC with a photoperiod of 12 h for 10 days. The conidia suspension (Figure 1C) was obtained by scraping the sporulation cultures (Figure 1B) using sterile distilled water with a drop. L^{-1} of Tween 20 and subsequent adjustment to 10^{4} conidia. mL^{-1} with a Neubauer chamber, for the inoculation of *R. pseudoglycines,* A mixed suspension with equal parts (1:1:1) of conidia suspensions from two different isolates were prepared.

Thirty days old plants (Figure 1 D) were inoculated using a hand sprayer. Immediately after inoculation, the plants were incubated in a dark growth chamber adjusted to 21ºC and relative humidity of approximately 100% for 24h (ZANDONÁ et al., 2012). After this period, the plants were transferred to a greenhouse bench and randomized.

3.2 Light Microscopy and Histochemical Tests

Leaf tissue collections were performed 12 and 48 hours after inoculation (hai), in addition to the incubation period observed for each cultivar. Leaf fragments measuring 0.5 × 0.5 cm from the mid-central region between the midrib and the edge of the blade were collected and transferred to microtubes with karnovsky fixative solution.

For blocking in Historesin (Leica ®), the samples were initially dehydrated in 70, 80, 90 and 100% ethyl alcohol. Afterwards, the infiltration process was carried out, with activated resin: ethanol (1:1) for 48 h, followed by 100% activated resin for 96 h. The samples were placed in histomolds with inclusion resin, where they should remain incubated for 24 h at 50 ºC. A rotating microtome was used to obtain slices about 5 µm thick from the blotted samples.

Phenolic compounds were detected in plant tissues as described by (MARQUES AND SOARES, 2022) and the imagens were obtain using an Epi-Fluorescence microscope Carl Zeiss MicroImaging Z1 (Carl Zeiss, Göttingen, Germany).

3.3 Scanning Electron Microscopy (SEM)

Leaf tissue collections were carried out at 6, 12, 24, 48 hai, in addition to the incubation period (IP) that is observed for each cultivar (Figure 1 E). Fragments of leaves measuring 0.5 × 0.5 cm from the mid-central region between the midrib and the edge of the blade were collected and transferred to microtubes with Karnovsky fixative solution (glutaraldehyde 2.5%, formaldehyde 2.5% and $CaCl_2$ 0.1M in 0.05M sodium cacodylate buffer pH 7.5).

After the minimum time of 24 hours of fixation, the preparation of samples for observation in SEM started with washing in 0.05M sodium cacodylate buffer pH 7.5 for 3 times of 10 minutes. Then, dehydration with solutions of increasing concentration of acetone (24, 50, 75, 90 and 100% three times, 10 minutes each), and drying using a critical point device (BOZZOLA; RUSSELL, 1999; ALVES et al., 2013). Afterwards, assembly in aluminum sample holders (*stubs*), metallization with gold and observation in a Scanning Electron Microscope Tescan-Clara (Germany).

3.4 Disease Assessment

The severity of RLS were evaluated for 21 days with intervals of three days from the onset of the first symptoms using the diagrammatic scale proposed by Aquino et al., (2008). The scores obtained were interpolated for severity values, in percentage. Then, the estimated severity values were integrated as the area under the disease progress curve (AUDPC), through the formula $AUDPC = \Sigma \{[(y1 + y2)/2] * (t2 - t1)\}$, where y1 and y2 are two consecutive severity assessments performed at times t1 and t2, respectively, as proposed by Shaner & Finney, (1977).

3.5 Statistical Analyses

The experimental design was in randomized blocks with three treatments (three cotton genotypes) with five replications (blocks) and maximum of five collections each over time, totaling 30 plots. Each plot consists of a pot with two plants. The experiment was repeated once.Analysis of variance (ANOVA) and Scott-Knott test (at 5% probability) were performed to compare the means between treatments, for each pathogen and for each time evaluated, using software R and package Agricolae.

4. RESULTS

4.1 Area Under the Disease Progress Curve (AUDPC)

Among the two isolates studied, isolate (B) provided the highest AUDPC when inoculated in the susceptible cultivar (D), statistically differing from the other interactions

between cultivars and isolates, which showed the lowest AUDPC (Figure 1). Given this observation (greater aggressiveness of the isolate (B)), the microscopic analyzes presented in this study were performed with this isolate.

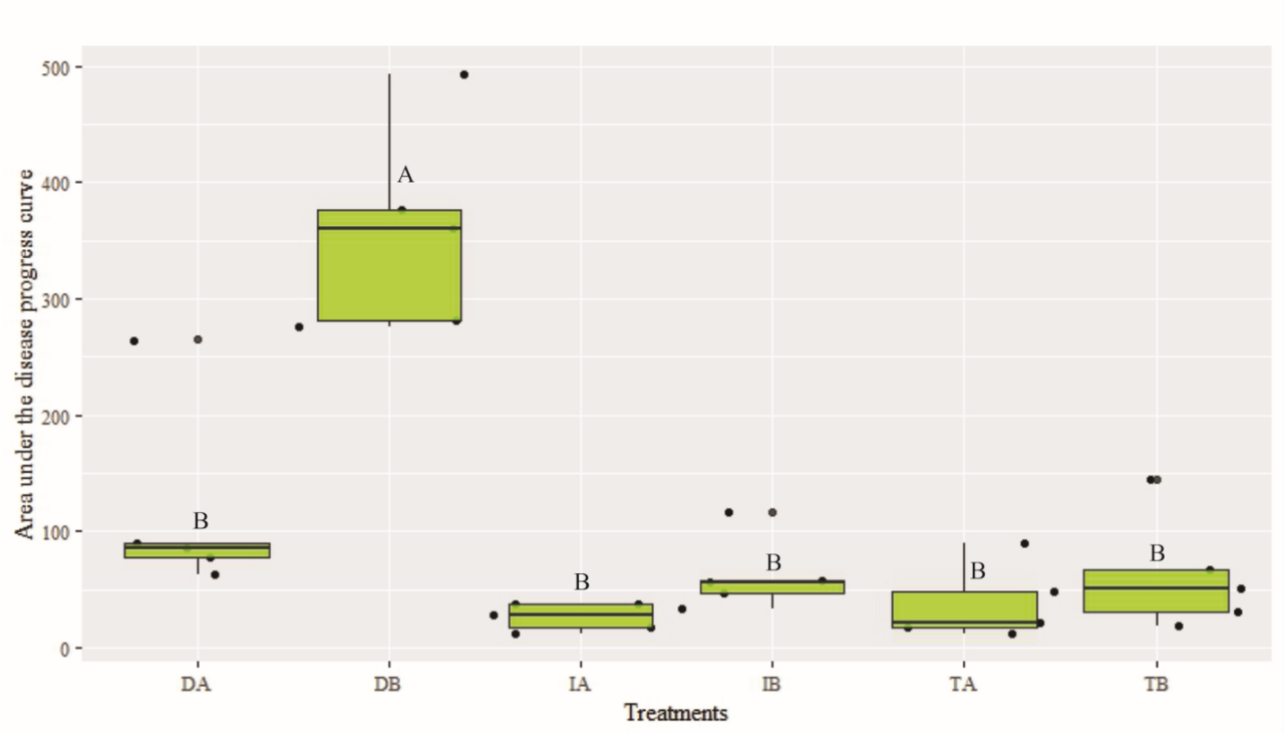

Figure 1 - Boxplot distribution of Ramularia Leaf Spot (RLS) Area Under Disease Progress Curve (AUDPC) values in three cotton cultivars with different levels of resistance the IMA 5801 B2RF (highly resistant) (I), DP1746 B2RF (high susceptibility) (D), TMG44 B2RF (moderately resistant) (T) inoculated with two isolates of *R. pseudoglycines* (FAL F3 (A) e FAL FICC (B)). Means followed by the same letter are not significantly different by *Scott-Knott* test at $p \leq 0.001$. The error bars in each box represent the average standard error of the AUDPC values.

4.2 Light Microscopy and Histochemical Test

A significant accumulation of phenolic compounds was observed in the cultivar TMG 44 (moderately resistant) (T), evidenced by the intense dark yellow and dark brown color (Figure 2 C). In the cultivar IMA 5801 (highly resistant) (I), less dark brown areas were observed in relation to the cultivar TMG 44 (moderately resistant) (T), but the dark yellow color continued to be very evident (Figure 2 B). Only in cultivar DP 1746 (high susceptibility), the intensity of the yellow color is much less evident and the dark brown color is not observed, indicating a low accumulation of phenolic compounds (Figure 2A).

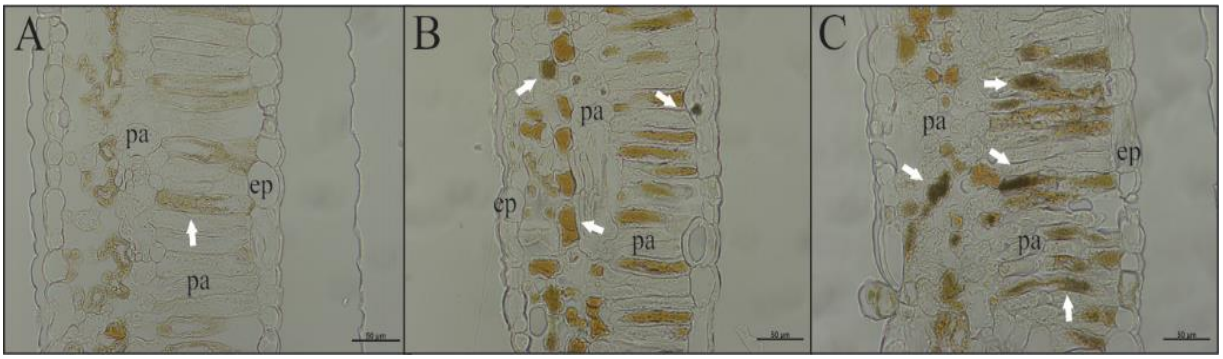

Figure 2 – Photomicrographs showing the production of phenolic compounds after of the infection of the FAL F1CC (B) isolate of *Ramulariopsis pseudoglycines* in three cotton cultivars with three levels of resistance: **A** - DP 1746 (high susceptibility) (D); **B** - IMA 5801 (highly resistant) (I); **C** - TMG 44 (moderately resistant) (T) at 24 hours after inoculation (hai). The arrows indicate the accumulation of phenolic compounds evidenced by the dark yellow and dark brown coloration after treatment with ferric chloride dye; **ep** and **pa** indicate epidermis and parenchyma, respectively. The scale bars in **A**, **B** and **C** correspond to 50 µm.

4.3 Scanning Electron Microscopy (SEM)

In the SEM analyses, the initial hours of the infectious process can be observed through the germination pattern of the conidia in relation to the different cultivars. At 12 hai, germination and penetration via conidia stomata occurred in the DP 1746 cultivar (high susceptibility), whereas for the other cultivars, few germinated conidia and poorly developed twins were found (Figure 3 A, B and C). During the infectious process, this pattern of conidia germination was observed. In the 96h cultivar IMA 5801 (highly resistant), it was still possible to observe non-germinated conidia, different from the DP 1746 (high susceptibility) and TMG 44 (moderately resistant) cultivars, where the amount of germinated conidia on the cotton leaf surface was higher (Figure 3 M, N and O), respectively.

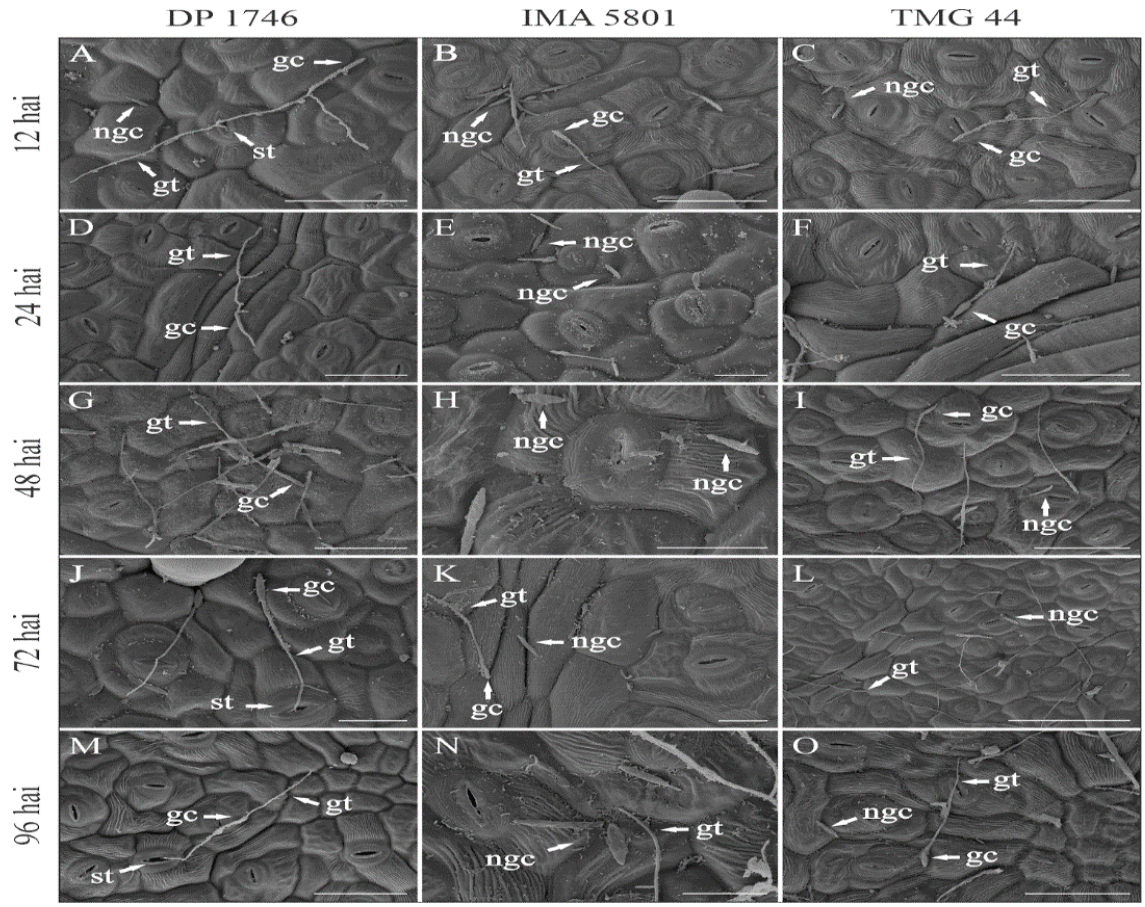

Figure 3 - Scanning electron micrographs of the infection of the FAL F1CC (B) isolate of *Ramulariopsis pseudoglycines* in three cotton cultivars with three levels of resistance: DP 1746 B2RF (high susceptibility) (D); IMA 5801 B2RF (highly resistant) (I); TMG 44 B2RF (moderately resistant) (T) at 12, 24, 48, 72 and 96 hours after inoculation (hai). Abbreviation: gc – germinated conidia; ngc – not germinated conidia; gt – germ tube; st – stomata; hai – hours after inoculation. Bars in E, H, J, K, N and O = 20 µm; A, B, C, D, F, G, I and M = 50 µm; L = 100 µm.

5. DISCUSSION

The result shows three different cotton genotypes with different resistance and susceptibility to ramularia leaf spot conducted at UFLA green house. Among the treatments DB (357.3) is the one with high susceptibility, while IA (26.4) has more resistant and TB (62.1) is moderately resistant to *Ramularia pseudoglycines*. This corresponds with the finding of Pedrosa et. al., (2020), which indicates that DP 1746 cultivars are more susceptible, IMA 5801 cultivars are high resistance and TMG 44 cultivars are moderately resistance. Fungicides are widely used for RLS control in Brazil. Some of them have been very effective in reducing progress of RLS and consequently, partially protecting yield. However, given the high cost of the applications (product price, fuel and labor), as well as environmental impact, research data on the comparative performance, number and timing of fungicide sprays are scarce. Additionally, concerns about the emergence of isolates that are insensitive to the main fungicides, or chemical group, should be considered and further investigated given the high number of fungicide sprays applied during the season (SUASSUNA AND COUTINHO, 2014). Triazole, strobilurin, carboxamides and tri-fenil tin-based fungicides have shown effective for RLS management in Brazil (SUASSUNA AND COUTINHO, 2015).

Aquino et al., (2008) evaluated the efficacy of protectant fungicides including the "Viçosa mixture" (a nutrient amended Bordeaux mixture), mancozeb and chlorothalonil. Currently, a network of cooperative trials for evaluation of fungicides in controlling RLS identified the following fungicides (or combinations) with the best performance in preventing fiber yield losses: piraclostrobin + fluxapiroxade, carboxamide + copper oxychloride, trifloxystrobin + prothioconazole + bixafen, azoxystrobin + difenoconazole + chlorothalonil, fentin hydroxide, mefentrifluconazole, isofetamid, chlorothalonil, azoxystrobin + difenoconazole, mancozeb, chlorothalonil, and piraclostrobin + metconazole (ARAÚJO et al., 2019), so the resistant cultivar is a good strategy to management of this disease.

In RLS it has been reported that cotton may present either immune reactions or compatible ones with early necrosis, compatible with late necrosis and hypersensitivity reaction. Resistant cultivars are the cheapest and most efficient method for RLS disease control (RATHAIAH, 1976). This is in line with my findings after inoculation in the green house, the leave start with chlorosis, necrosis and hypersensitive reaction in some leave. There is variation in the degree of susceptibility to RLS found in the different cultivars belonging to the genus Gossypium (*G. hirsutum, G. barbadense and G. arboretum*) and, in countries where the disease

is endemic and economically damaging, the best control should be the use of resistant cultivars. It is possible to generate segregating populations and select genotypes with desirable agronomic characteristics and resistance to RLS. However, until recently, cotton breeding programs in Brazil have focused mainly on high yield potential, high fiber quality and resistance to diseases of monogenic inheritance (bacterial blight and cotton blue disease), thus, most of the cultivars released in the past years are susceptible to RLS. Consequently, generation of widely-adapted high-yielding cotton cultivar that exhibit resistant to the main diseases is a great challenge for the breeders. Identification of resistance sources and knowledge about the resistance inheritance are needed in order to develop new resistant cultivars. Several studies were performed aiming to screening resistance sources to RLS. Ascari et al., (2016) showed the cultivar FMT 705 was less susceptible to RLS, followed in order of increasing susceptibility by the cultivars FMT 709, IMACD 8276 and FM 951LL. However, with this recent development of identifying most resistant cultivar in this research, it will help farmers to increase their cotton production in Brazil and across the globe.

The biopesticides are an alternative to sustain high production with low ecological impact. Research in the development of biocontrol is currently expanding exponentially (PÉREZ et al., 2015) with Trichoderma-based products the most important biofungicides used worldwide (LORITO et al., 2010). According to Bettiol et al. (2014), Trichoderma sp. is the most important commercialized biocontrol agent and is used for controlling soil borne, foliar and postharvest diseases in several crops (HARMAN et al. 2004; HARMAN 2006; WOO et al., 2006). This biocontrol agent protects plants by producing antibiotics and enzymes, promoting plant growth, and inducing plant defenses, in addition to increasing competition for nutrients (HERMOSA et al., 2012). Biofungicides efficiently control plant diseases and are environmentally safe; however, although their use is expanding annually, the adoption by growers remains slow (BETTIOL, 2011). In cotton, the foliar application of *Trichoderma asperellum* (SILVA et al., 2017) reduced the severity of RLS when sprayed alone or in combination with a fungicide. The use of Silicon (Si) as a resistance inductor on cotton increases lignin content and enzymes activities (POX, PFO, QUI, GLU and FAL) in highly susceptible cultivar NuOpal and increases enzymes activities (POX, QUI e GLU) in partial resistant cultivar BRS 269-Buriti under high disease pressure (CURVÊLO et al., 2013). Applications of Acybenzolar-s-methyl at the rate of 20 g.i.a ha−1 reduced the severity of the disease (MARTINS et al., 2015).

Crop rotation, certified seeds, elimination of volunteer plants and stalk regrowth from the previous season, soil tillage, sowing recommend period, plant density, adequate plant nutrition and others are the main cultural practices (BEDENDO et al., 2011). The density and the plant height also are related to increase the severity of the disease, because, taller plants and high population can create a favorable environment to the disease development, due to shadowing and humidity accumulation in the lower part of the plant (SUASSUNA and COUTINHO, 2015).

6. CONCLUSIONS

Base on the research conducted previously by so many researchers, it was discovered that the use of resistant cultivar is the cheapest and most efficient method of RLS control. The production of phenolic compounds as plant defense mechanisms may have contributed in the TMG 44 (moderately resistant) and IMA 5801 (highly resistant) cultivars, to the lower AUDPC observed, in relation to the DP 1746 (high susceptibility) cultivar. The susceptible cultivar DP 1746 B2RF demonstrated Low phenolic compounds, High conidia germination, Highest AUDPC in contrast with the other two resistant genotypes. The resistant cultivars IMA 5801 B2RF and TMG 44 B2RF, demonstrated low conidia germination, low *R. pseudoglycines* penetration via stomata and low AUDPC.

7. REFERENCES

Adams GM (2015) Marketing and economics. In: Fang DD, Percy RG (Eds.) cotton. 2nd ed. agronomy monograph 57. ASA, CSSA, and SSSA, Madison.

Alves, E.; Lucas, G. C.; Pozza, E. A.; De Carvalho Alves, M. (2013) Scanning Electron Microscopy for Fungal Sample Examination. In: Laboratory Protocols in Fungal Biology. [s.l.] Springer, 2013. p. 133–150.

Aquino, L. A.; Berger, P. G.; Rodrigues, F. Á.; Zambolim, L.; Hernandez, J. F. R.; Miranda, L. M. (2008) Elaboration and validation of a diagrammatic scale to quantify areolate mildew on cotton. Summa Phytopathologica, v. 34, p. 361–363.

Atkinson GF (1890). A new Ramularia on cotton. Botanical Gazette 15:2 166–168.

Barros R, Degrande PE, SORIA MF, Ribeiro JSF (2008) Ocorrência de manchas foliares causadas por fungos e bactérias em cultivares de algodoeiro. Pesquisa Agropecuária Tropical 38:297–303.

Bell AA (1981) Areolate mildew. In: Watkins GM (ed) Compendium of cotton diseases. The American Phytopathological Society, St Paul, 87 pp

Braun U (1993) Studies on Ramularia and allied genera (VI). Nova Hedwigia 56:423–454

Bozzola, J. J.; Russell, L. D. (1999) Electron microscopy: principles and techniques for biologists. [s.l.] Jones & Bartlett Learning.

Caplan, J. L.; Kumar, A. S.; Park, E.; Padmanabhan, M. S.; Hoban, K.; Modla, S.; Czymmek, K.; Dinesh-Kumar, S. P. (2015) Chloroplast stromules function during innate immunity. Developmental cell, v. 34, n. 1, p. 45–57.

Cauquil J, Sément GL (1973) False mildiou du cotonnier (Ramularia areola Atk.) dans le sud-ouest de Madagascar. Coton et Fibres Tropicales 28:279–286.

Cia E (1977) Ocorrência e conhecimento das doenças de algodoeiro anual Gossypium hirsutum L no Brasil. Summa Phytopathologica 3:167–193.

Cia E, Salgado CL (1997) Doenças do algodoeiro. In: Kimati H, Amorim L, Bergamin Filho A, Camargo LEA, Rezende JAM (Eds.) Manual de Fitopatologia. Vol. 2 Doenças de Plantas Cultivadas. 4 Ed. Ceres, São Paulo. pp. 33–48.

Curvêlo, C. R. da S.; Rodrigues, F. Á.; Silva, L. de C.; Nascimento, K. J. T.; Berger, P. G. (2013) Biochemical aspects of cotton resistance to ramularia leaf spot mediated by silicon. Bragantia, v. 72, p. 41–51.

Curvelo, C. R. S.; Rodrigues, F. A.; Berger, P. G.; Rezende, D. C. (2010) Scanning microscopy of the infectious process of Ramularia areola on cotton leaves. Tropical Plant Pathology, v. 35, p. 108–113.

DA Silva, J. C.; Bettiol, W.; Suassuna, N. D. (2019) Ramularia leaf spot: an emergent disease of cotton in Brazil. Tropical Plant Pathology, v. 44, n. 6, p. 473–482.

Daudi, A.; O'brien, J. A. (2012) Detection of hydrogen peroxide by DAB staining in Arabidopsis leaves. Bio-protocol, v. 2, n. 18, p. e263–e263.

Ehrlich J, Wolf FA (1932) Areolate mildew of cotton. Phytopathology 22: 229–240

Ferreira Filho JBS, Alves LRA (2007) Aspectos econômicos do algodão no cerrado. In: Freire EC (ed) Algodão no Cerrado do Brasil. Associação Brasileira dos Produtores de Algodão, Brasília, pp 53–89.

Galbieri, R.; Cia, E.; Morello, C. de L.; Fanan, S.; Andrade Junior, E. R.; Kobayasti, L. (2015) Ramularia areola sporulation potential in Brazilian cotton. Summa Phytopathologica, v. 41, p. 233–235.

Gows MA, Prinsloo GC, Van Der Linde EJ (2001) First report of Mycosphaerella areola, teleomorph of Ramulariopsis gossypii, on cotton in South Africa. African Plant Protection 7:115–116

Johansen, D. A. (1940) Plant microtechnique. Plant microtechnique., n. First Ed.

Marques, J. P. R.; Hoy, J. W.; Appezzato-da-Glória, B.; Viveros, A. F. G.; Vieira, M. L. C.; Baisakh, N. (2018) Sugarcane cell wall-associated defense responses to infection by Sporisorium scitamineum. Frontiers in Plant Science, v. 9, p. 698.
Marques, J. P. R.; Soares, M. K. M. (2022) Handbook of Techniques in Plant Histopathology. [s.l.] Springer Nature.

Mathioni, S. M.; De Mello, F. E.; Antunes, R. F. D.; Duvaresch, D. L.; Milanesi, D. F.; Brommonschenkel, S. H.; Pinho, D. B.; Rosa, D. D. (2021) Species Determination and CYTB-G143A Monitoring of Ramulariopsis spp. Isolated from Cotton in Brazil. Plant Health Progress, v. 23, n. 1, p. 4–6.

Mehta YR, Menten JOM (2006) Doenças e seu controle. In: Moresco E (ed) Algodão – Pesquisas e Resultados para o Campo, vol 2. FACUAL, Cuiabá, pp 157–205.
Moreira, S. I.; Pereira, L. F.; Souza, E. A. de; Alves, E. (2022) Fluorochrome-Based Methods for

Fungal Sample Examination. In: Laboratory Protocols in Fungal Biology. [s.l.] Springer, 2022. p. 209–234.

Morello CL, Suassuna ND, Barroso PAV, Silva JL, Ferreira ACB, Lamas FM, Pedrosa MB, Chitarra LG, Ribeiro JL, Godinho VPC, Lanza MA, (2015) BRS 369RF and BRS 370RF: glyphosate tolerant, highyielding upland cotton cultivars for central Brazilian savanna. Crop Breeding and Applied Biotechnology 15:290–294.

Morello, C. L.; Shuster, I.; Mehta, Y. R. (2012) Mechanism of resistance and presence of different resistance genes to Ramularia areola in two cotton genotypes. Tropical Plant Pathology, v. 37, p. 175–178.
Pedrosa, M. B.; Eleusio, B.; Freire, C.; Consultoria, C.; Desde, A. (2020) Resultados das primeiras avaliações realizadas no projeto adoção de novas cultivares de Algodoeiro no cerrado da Bahia - Safra 2020 / 21.

Pezenti, L. F.; Barbosa, J.; Vieira, M. A.; Marangoni, M. S.; Volponi, J.; Almeida, W. P.; Galbieri, R.; Mehta, Y. R. (2013) Phenotypic variability among isolates of Ramularia areola from Brazilian cotton. Tropical Plant Pathology, v. 38, p. 329–331.

RATHAIAH, Y. (1976) REACTION OF COTTON SPECIES AND CULTIVARS TO FOUR ISOLATES OF RAMULARIA AREOLA.

Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B. Fiji: (2012) an open-source platform for biological-image analysis. Nature methods, v. 9, n. 7, p. 676–682, 2012.

Shaner, G.; Finney, R. E. (1977) The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology, v. 67, n. 8, p. 1051–1056.
Shivankar SK, Wangikar PD (1992) Estimation of crop losses due to grey mildew disease of cotton caused by Ramularia areola. Indian Phytopathology 45:74–76.

Silva JC, Suassuna ND, Bettiol W (2017) Management of Ramularia leaf spot on cotton using integrated control with genotypes, a fungicide and Trichoderma asperellum. Crop Protection 94:28–32

Silva, A. S.; Renno, M. H. L.; Quitania, A. C. R.; Café-Filho, A. C.; Miller, R. N. G.; Araujo, A. E.; Pinho, D. B. (2021) Ramularia leaf spot: PCR-based methods reveal widespread distribution of *Ramulariopsis pseudoglycines* and limited presence of R. gossypii in Brazil.

Spegazzini C. (1886) Fungi Guaranitici: Cercosporella gossypii Speg (n sp). Anales de la Sociedad Científica Argentina 22:209

Suassuna ND, Coutinho WM (2014) Manejo de doenças. In: Borém a, Freire EC (Eds.) Algodão: do plantio à colheita. 1Ed. Editora UFV, Viçosa, pp 250–270.

Suassuna ND, Coutinho WM (2015) Manejo das principais doenças do algodoeiro no cerrado brasileiro. In: Freire EC (ed) Algodoeiro no Cerrado do Brasil. 3 Ed. Gráfica e Editora Positiva, Brasília, pp 365– 408.

Tormen, N. R.; Blum, L. E. B. (2019) Ramularia leaf spot effect on yield and fiber quality of cotton submitted to fungicide application. Revista Caatinga, v. 32, p. 634–646.
Videira SIR, Groenewald JZ, Braun U, Shin HD, Crous PW (2016) All that glitters is not Ramularia. Studies in Mycology 83:49–163

Wendel JF, Grover CE (2015) Taxonomy and evolution of the cotton genus, Gossypium. In: Fang DD, Percy RG (Eds.) Cotton. 2nd. ed. agronomy monograph 57. ASA, CSSA, and SSSA, Madison.

Yin, X.; Liu, R.-Q.; Su, H.; Su, L.; Guo, Y.-R.; Wang, Z.-J.; Du, W.; Li, M.-J.; Zhang, X.; Wang, Y.-J. (2017) Pathogen development and host responses to *Plasmopara viticola* in resistant and susceptible grapevines: an ultrastructural study. Horticulture Research, v. 4.

ZANDONÁ, C.; NOVAES, T. G.; NUNES, M. P.; ALMEIDA, W. P.; AGUIAR, P. H.; Morello CL, Shuster I, Mehta YR (2012) Mechanism of resistance and presence of different resistance genes to Ramularia areola in two cotton genotypes. Tropical Plant Pathology 37:175–178