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Mycosphaerella areola—The Teleomorph of *Ramularia areola* of Cotton in Brazil, and Its Epidemiological Significance

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Abstract

While Ramularia leaf blight of cotton caused by *Ramularia areola* is of top most importance for Brazil, information is lacking regarding the survival mechanism of this pathogen during the cotton-free period. The teleomorph of *R. areola* is expected to belong to the genus *Mycosphaerella*. In the present study attempts were made to verify occurrence of this teleomorph in the State of Mato Grosso, Brazil. Decaying cotton leaves were collected two months after harvest of 2014 from 44 commercial and experimental fields where aerial fungicidal applications were made or not during the crop cycle to control the Ramularia leaf blight. Examination of the decaying cotton leaves revealed presence of abundant sclerotia, spermagonia and ascoma of *Mycosphaerella* sp. intermingled with each other during the cotton-free period in most of the leaf samples. Mono-ascospore isolations were obtained from the ascoma and considering their cultural, morphological, pathological and DNA sequence analysis they were identified as *Mycosphaerella areola*. *M. areola* and *R. areola* isolates produced similar symptoms under glasshouse inoculations. Reisolation of the pathogen from the symptoms produced by *M. areola* isolates yielded *R. areola*. Some cotton leaves showing such symptoms were kept on the soil surface on plastic trays for two months under natural field condition. After this period the decaying leaves showed abundant perithecia identical to their original *M. areola*. ITS rDNA sequence analyses revealed identical sequences from *M. areola* and *R. areola* isolates. Occurrence and the viability of the perfect stage *M. areola* during the cotton-free period on the left-over stubble from one season to another were interpreted as the survival mechanism of the pathogen and were considered responsible for the Ramularia blight epidemics in the State of Mato Grosso. Disease management practices for the State of Mato Grosso are

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discussed. This is the first report about the occurrence of the *M. areola* in Brazil.

Keywords

***Gossypium hirsutum*, Ramularia Leaf Blight, Sexual Morph, ITS rDNA Sequence Analysis**

1. Introduction

Ramularia leaf blight caused by *Ramularia areola* Atk. is an important disease in several cotton producing countries [1]. It is an endemic disease in the State of Mato Grosso, Brazil [2]-[4]. At present, the disease is partially controlled by as many as 5 - 11 fungicidal applications during the crop cycle which is not eco-friendly and in due course of time may not be sustainable. Limited information is available regarding the epidemiological aspects of this disease. Physiologic specialization has not been reported in this pathogen in Brazil. Recently, Girotto *et al.*, 2013 [5], reported genotypic and phenotypic variability within the isolates of *R. areola* collected from different geographical regions of Brazil, but failed to find relationship between these variables. While no information was available regarding the occurrence of teleomorph (sexual stage of *R. areola*) in Brazil, existence of "field strains" was believed to be present [5].

Mycosphaerella spp. can be saprobic or parasitic and had been linked to over 27 anamorph genera [6]. The teleomorph of *R. areola* possibly belonging to *Mycosphaerella areola* was first reported as early as in 1932 by Ehrilch and Wolf [7], although these authors could not conclusively demonstrate its relationship with the anamorph *R. areola*. Gouws *et al.* [8] reported relationship between *R. areola* and *M. areola* by pathogenicity tests under glasshouse conditions. So far, no information is available regarding the survival mechanism of this pathogen during the cotton-free period in Brazil. The objectives of the present investigation, were to verify the occurrence of teleomorph of *R. areola* in the State of Mato Grosso and to study its relationship with the anamorph, in order to comprehend its epidemiological significance.

2. Material and Methods

Leaf samples: Samples of decaying cotton leaves were collected two months after harvest from 31 experimental plots at two locations where no fungicide was applied and from 13 commercial cotton fields where 5 - 6 fungicidal applications were performed during the crop cycle in the State of Mato Grosso. Samples were brought to the laboratory and stored in dry atmosphere for further use.

Mono-ascosporic isolates: Leaf samples were examined under microscope for the presence of sexual forms (propagules) of the pathogen such as sclerotia, spermagonia and ascoma. Leaf portions were washed both sides with a jet of distilled water for several minutes to remove the soil particles and the fungal saprophytes. Later small leaf portions showing abundant ascomata were sterilized for one min in 70% alcohol followed by two min. in 1% sodium hypochlorite. Single ascospore isolations were made by fixing a small surface sterilized decaying leaf portion showing ascomata on the Petri dish lid so that the ascoma were facing a thin layer of water-agar in Petri dish containing 25 $mg \cdot L^{-1}$ of streptomycin. The Petri dish lids were rotated every few hours to obtain uniform distribution of ascospores. After incubation at 21˚C for 18 - 20 h the dishes were examined under microscope and the well separated germinating ascospores were rescued on V8 juice-agar. Three monoascosporic isolates were obtained.

Mono-pustular isolations: Monopustular isolations of *R. areola* were made as reported earlier [4] [5] by picking up conidia by a fine needle from the sporulating lesions and placing them on Petri dishes containing a thin layer of water agar. Since the needle may not pick up only one conidium the isolation is referred as "monopustular".

Pathogenicity test: Pathogenicity tests of *Mycosphaerella* isolates were conducted in the glasshouse on susceptible cotton cultivars IMACD 6001LL and DeltaOpal. The inoculation procedure was the same as described earlier [4] [5]. Plants inoculated with an aggressive isolate of *R. areola* no. 44 from culture collection of IAPAR, served as control, where suspension of the spores was adjusted to 10^4 $spores \cdot ml^{-1}$. After three weeks of inoculation reisolations of the pathogen were made from the leaf symptoms.

Molecular analyses: Genotypic variation among one *Mycosphaerella* sp. (MA1245-1) and three *R. areola*

isolates (RA1403019, RA44 and RA17.5) was studied using sequence analysis of internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). Genomic DNA was extracted as described in earlier studies [5].

All four isolates were analyzed by sequencing the ITS rDNA region (ITS4—5'-TCC TCC GCT TAT TGA TAT GC-3'; ITS5—5'-GGA AGT AAA AGT CGT AAC AAG G-3'). The amplification products of the ITS1 rDNA region were sequenced in an automatic sequencer AB1 Prism 3700 DNA Analyzer (Applied Biosystems Inc.). The PCR reaction procedure for ITS were: Stage 1—1 cycle: 94˚C for 3 minutes, Stage 2—35 cycles: 94˚C for 1 minute, 55˚C for 1 minute, 72˚C for 2 minutes, Stage 3—1 cycle: 72˚C for 5 minutes and 4˚C unlimited.

The ITS rDNA sequences obtained for the isolates RA1403019, RA44, RA17.5 and MA1245-1 were deposited in NCBI database under the accession numbers KR265336, KR265338, KR265337 and KX356661, respectively.

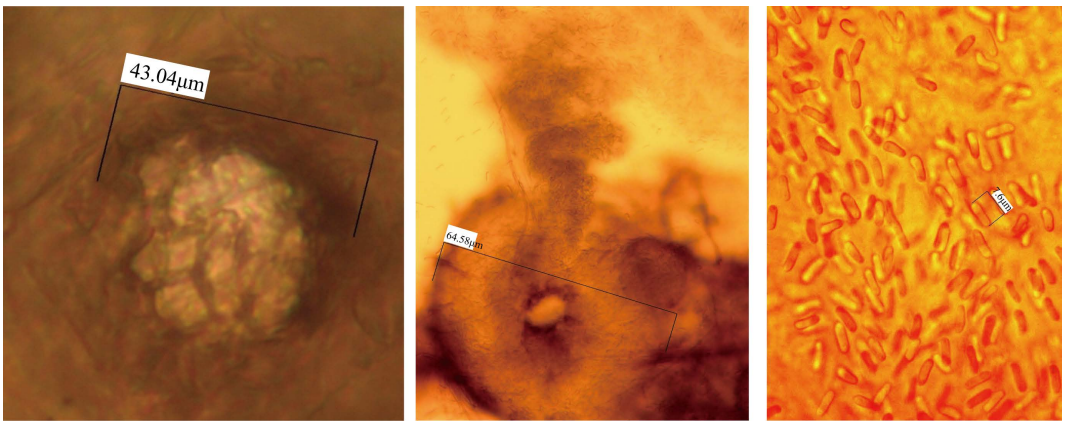
3. Results

Examination of the decaying cotton leaves on left-over stubble revealed the presence of abundant sclerotia, spermagonia and ascoma intermingled with each other on both the leaf surfaces in most of the field samples collected from 4 locations irrespective whether fungicides were sprayed or not during the crop cycle (**Table 1**). A total of 44 leaf samples were examined. The occurrence of teleomorph in two locations (Primavera do Leste and Campo Verde) is compared. In Primavera do Leste it was 71.41% and in Campo Verde it was 28.59%.

Spermagonia were between 30 - 65 µm and released numerous rod-shape spermatia (**Figure 1**). The spermatia were up to 7.6 µm in length and germinated within 6 - 8 hours in distilled water with 2% glucose. However, the role of spermatia in the infection process is not known. Typical asexual conidia of *R. areola*, were also found on some of the decaying leaves together with ascoma.

Table 1. Number of left-over decaying cotton leaf samples collected from four locations two months after harvest showing presence or absence of ascoma of *Mycosphaerella* sp., Mato Grosso, Brazil, 2014.

Location	Number of decaying leaf samples showing presence or absence of ascoma of *Mycosphaerella* sp.	
	Presence	Absence
1. Primavera do Leste (commercial fields with 5 - 6 fungicidal applications)	4	2
2. Campo Verde (commercial fields with 5 - 6 fungicidal applications)	6	1
3. Primavera do Leste (experimental fields without fungicidal applications)	16	0
4. Campo Verde (experimental fields without fungicidal applications)	2	13



(a) (b) (c)

Figure 1. *Mycosphaerella* sp. sexual morphs on decaying cotton leaf. (a) Sclerotia (43.04 µm); (b) spermagonia (64.58 µm) releasing spermacia; (c) rod shape spermacia (7.6 µm).

Ascoma of *Mycosphaerella* sp. were 40 - 60 µm in diameter (**Figure 2**). They contained several hyaline, bitunicate asci with a distinct foot cell. The size of the asci varied between 54.1 and 67.8 µm in length (**Figure 3**). Each ascus contained one septate eight ascospores, showing constriction at the septum. The ascospores were hyaline, measured 6.6 × 18.2 µm and germinated by both cells (**Figure 4(a)**). These fruiting bodies resembled *Mycosphaerella areola* as described by Ehrlich and Wolf [7] and hence here onwards they are referred as *M. areola*.

Out of three *M. areola* isolates one was not pathogenic under greenhouse inoculations on susceptible cotton cultivar. Failure to show pathogenicity by one of the *Mycosphaerella* isolates is probably due to their saprophytic nature. Saprobic *Mycosphaerella* spp. in nature is already known [6]. All the three monopostular isolates

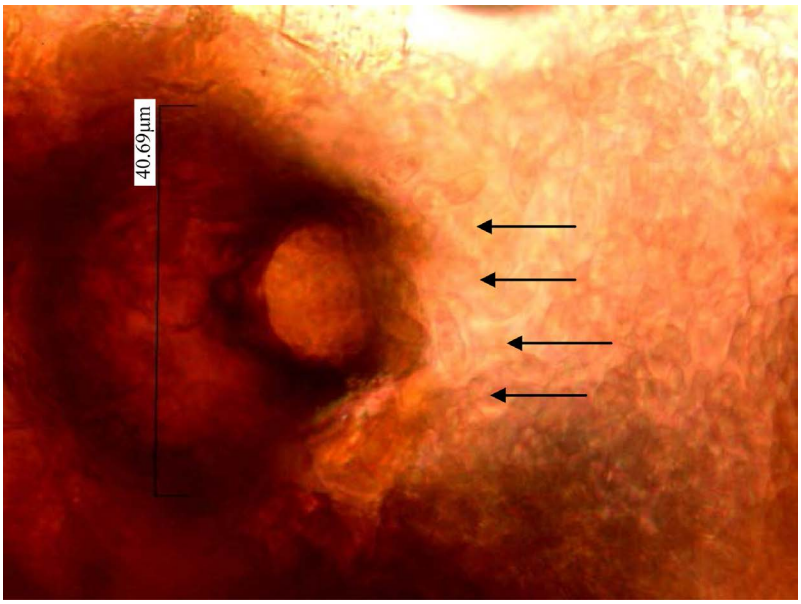


Figure 2. *M. areola*. Mature perithecium (40.69 µm) releasing ascospores indicated by arrows.

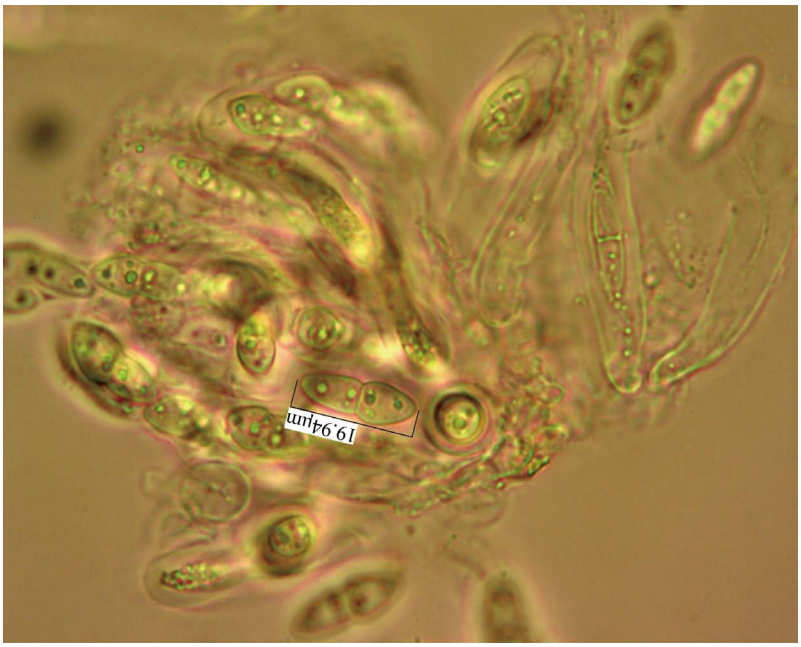


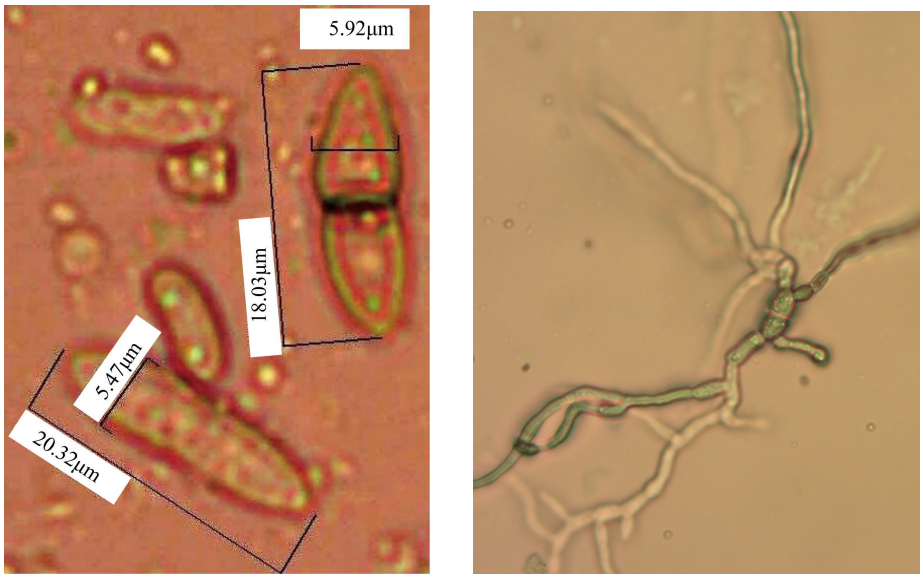
Figure 3. *M. areola*. Bi-tunicate asci with ascospores (19.94 µm).

of *R. areola* were pathogenic.

Leaf portions showing typical Ramularia symptoms produced after inoculation with *M. areola* isolates were incubated in a moist chamber for ten days. Typical conidia were produced within 48 hours and later within 7 - 8 days mature perithecia of *M. areola* similar to the original isolate were produced. Greenhouse inoculations made with the mono-ascospore isolates produced typical Ramularia blight symptoms three weeks after inoculation on susceptible cotton cultivars IMACD 6001LL and DeltaOpal (**Figure 5**). From such sporulating Ramularia symptoms monopustular isolates yielded typical colonies of *R. areola* on V8-juice agar (**Figure 6**).

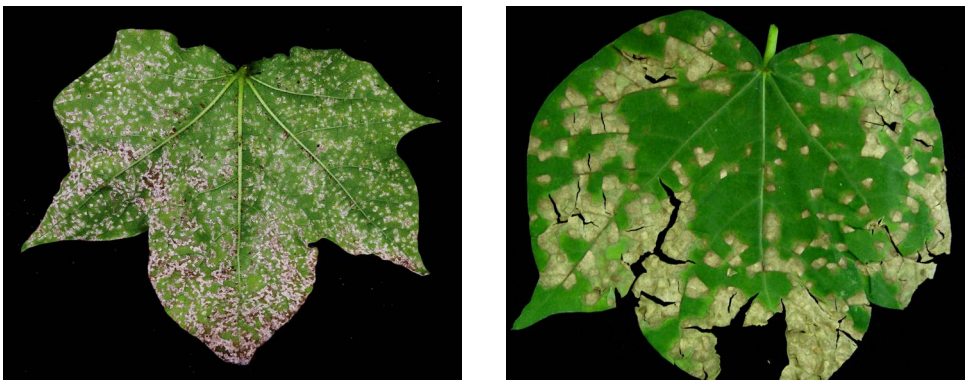
The ITS rDNA sequence analysis of the *Ramularia* isolates showed 99% identity to *Mycosphaerella areola*, confirming their relatedness.

Two types of ascoma were observed on the decaying leaves, one was mature, scattered, and was slightly larger than the other immature ones, appearing in clusters. Three single ascospore isolates, originated from scattered ascoma appearing on decayed cotton leaves, when cultivated on V8 juice-agar yielded spermagonia and ascoma intermingled with each other in abundance within seven days. Ehrlich and Wolf [7] reported that single ascospore colonies developed the same type of growth as those isolated from conidia but no conidia or spermagonia



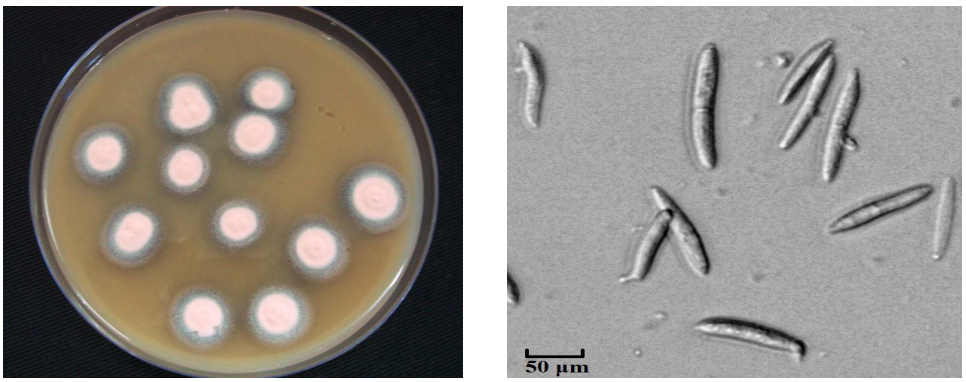
(a) (b)

Figure 4. *M. areola*. (a) Mature ascospores; (b) ascospore germination after 18 hours on water agar.



(a) (b)

Figure 5. *R. areola*. (a) Symptoms of Ramularia produced three weeks after inoculation of cv. IMACD 6001LL by two monoascosporic isolate of *M. areola*; (b) symptoms of Ramularia produced three weeks after inoculation on cv. DeltaOpal by monopustular isolate originated from symptoms produced by *M. areola* isolate (a).



(a) (b)

Figure 6. *R. areola.* (a) Colonies of monopostular isolates on V8 juice agar developed by rescuing *R. areola* conidia with a fine needle from the symptoms produced by *M. areola* isolate (see **Figure 5(a)**); (b) conidia.

were developed in these colonies. Gouws *et al.* [8] on the other hand, could find correlation between *Mycosphaerella* and *Ramularia* and reported that single ascospore cultures yielded pseudothecia but not the spermagonia.

Considering the cultural, morphological, pathological and the sequence analysis, our *Mycosphaerella* isolate was identified as *Mycosphaerella areola* Ehrlich and Wolf—the teleomorph of *R. areola* [7].

4. Discussion

Although *Mycosphaerella* is a complex genus, according to Crous *et al.* [6] it appears to be a monophyletic group. According to Hyde *et al.* [9], *Mycosphaerella* sensu *lato* is a valid applicable genus name currently considered a facultative (heterotypic) synonym of *Ramularia*, especially when its relationship with *R. areola* is not confirmed by its pathogenicity on cotton plants.

Our results confirm the relationship between the sexual form *M. areola* and the asexual form *R. areola* occurring in the State of Mato Grosso, Brazil. The occurrence of the perfect stage *M. areola* may be responsible for creation of new pathotypes of *R. areola*. It is believed that pathotypes or races of this pathogen may be present in Brazil and may be due to several processes, among them the sexual recombination due to the presence of *M. areola* which could play a major role. However, detailed studies in this respect are desired.

This is the first report of *M. areola* as the perfect stage of *R. areola* occurring naturally in Brazilian cotton fields in association with asexual form *R. areola*. We studied three monoascosporic *Mycosphaerella* isolates out of which only one was used for sequencing in order to prove its relationship with *R. areola*. Detailed pathological and molecular analyses with several *M. areola* isolates collected from different Brazilian cotton producing States are necessary to elucidate further the question whether *M. areola* is of common occurrence in other Brazilian States. In the interim, some integrated control measures for the disease can be practiced. The control of Ramularia blight cannot solely be dependent on fungicidal applications. Normally, cotton seeds are deslinted with sulfuric acid before sowing and hence so far there is no evidence about the seed transmission of this pathogen. In the field, the primary infection may come from ascospores as well as from the conidia produced on volunteer plants. The infection can also come from the secondary hosts as well but so far no secondary host of this pathogen has been reported.

Allied with pathological and molecular studies, aerobiological survey would be helpful to reveal the presence of ascospores during the cotton free period as well as during the sowing period of cotton [10]. The lack of presence of conidia of *R. areola* in the air during the initial stages of cotton crop for example, would indicate that the ascospores are solely responsible for the primary infection of the disease in the field. Normally, ascospores are wet spores, are heavy and are produced at the ground level and hence do not travel more than a few meters. Thus the secondary spread of the disease is through the air-borne conidia of *R. areola*. This would further emphasize the need to develop appropriate measures to eliminate soil-borne inoculum of *M. areola*.

The State of Mato Grosso is the largest cotton producing State, where at present 70% of Brazilian cotton is grown after the harvest of soybeans. After cotton harvest the left-over stubble is destroyed by obligatory local law to reduce the propagation of insect pests especially the cotton boll weevil—*Anthonomus grandis*. This oper-

ation is done either mechanically using mold-board plough and heavy disc harrow or chemically using, basically, 2,4 D and glyphosate herbicides. In the remaining 30% of the area cotton is grown in a monoculture system (cotton-cotton-cotton), where the left-over stubble is destroyed as explained before followed by planting millet (*Pennisetum glaucum*) as a cover crop. Later the millet crop is destroyed either by chemicals or incorporated by mechanical means and the cotton crop is sown again. Such operations drastically reduce the incidence of pests but do not destroy the propagules of the pathogens especially the ascomata of *M. areola* and thereby the pathogen completes its cycle when the next cotton crop is sown. Irrespective of the fact that in most of the area cotton is grown in sequence with soybean (soybean/cotton-soybean/cotton), it is still considered as a monoculture system. The monoculture system is practiced for several years in the same field and hence becomes responsible for sheltering the pathogen and consequently provoking severe epidemics of the disease year after year. As stated earlier, in all commercial fields irrespective of the system of cultivation, ascomata of *M. areola* were present on decaying cotton leaves (**Table 1**).

Besides being seriously condemned, stubble burning does not destroy completely the inoculum of the pathogen as evidenced in Australia and in Brazil for *Pyrenophora tritici-repentis* and for *Mycosphaerella graminicola* of wheat, and that a small undestroyed portion of inoculum may be sufficient to create an epidemic [11]-[13].

There exists serious concern among the cotton producers and the researchers to shift the traditional monoculture system either to minimum tillage or to direct drilling, or else to the integrated crop-livestock systems (agri-silvi-pasture). None-the-less, irrespective of any change in cultivation system the over-summering of *M. areola* as the sexual morph during the cotton free period seems to remain unaltered even more so for the 30% of the area spread in a mosaic form in the State, unless an intelligent and sustainable crop rotation system is also followed in the whole State and not in isolated areas. However, for this, further studies are necessary to verify the maximum period of survival of the sexual forms of *M. areola* in the left over stubble after cotton harvest. Cotton crop in rotation with soybean followed by some cover crops like *Brachiaria ruziziensis* or *B. brizantha* as non-hosts of *R. areola* could then be considered as some of the options. Allied with intelligent crop rotation, varietal resistance either through conventional breeding or through Marker Assisted Selection would undoubtedly constitute some of the most desirable measures to manage the disease.

5. Conclusion

1) Relationship between the sexual morph (*Mycosphaerella*) and the asexual morph (*Ramularia*) was conclusively demonstrated; 2) for the first time we report the occurrence of *M. areola* as the teleomorph of *R. areola* on decaying cotton leaves in the fields after harvest; 3) considering the fact that the cultivation systems currently used in the State of Mato Grosso harbors *M. areola* in the field from one season to another, use of intelligent crop rotation along with varietal resistance either through conventional breeding or through Marker Assisted Selection is suggested to manage the disease.

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