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Leaf Spot Severity in Forage and Seed-Transmitted *Exserohilum rostratum* in Different Plant Phenological Stages

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Abstract

In order to obtain a successful pasture management, it is essential to know mainly the material to be sown, the local edaphic characteristics, and the disease epidemiology in these areas. Besides identifying the causal agent, the quantification of damage caused by pathogens is essential for the adoption of effective control measures. Thus, the objective of this work was to study the severity and progress of *Exserohilum rostratum*. The fungus was inoculated at different phenological stages of *Brachiaria decumbens* cv. Basilisk and *Panicum maximum* cv. Mombasa. The transmission of this pathogen from infected plants to seeds was also evaluated. The pathogen was inoculated in the plants at the following stages: Vegetative I; Vegetative II; Booting I; Booting II; Reproductive I; and Reproductive II. Severity was evaluated using a grade scale ranging from no symptoms to plants with more than 50% of affected leaf area. In order to evaluate the transmission of *E. rostratum* in seeds produced by the inoculated plants, the blotter test was used. *E. rostratum* was pathogenic to *B. decumbens* cv. Basilisk and *P. maximum* cv. Mombasa. Basilisk plants were susceptible in all phenological stages, with higher severity from the Vegetative II stage and greater progress between the second and tenth days after inoculation, coinciding with the seed-plant transmission of the fungal inoculum from this point on.

Keywords: *Brachiaria decumbens, Panicum maximum*, Epidemiology, Helminthporporosis, Disease progress

Introduction

Brazil is a country that has a vast territorial extension and whose climatic conditions are excellent for the practice of agricultural activities [1]. With approximately 159 million hectares of pasture [2], the grasses *Brachiaria sp.* and *Panicum maximum* occupy a prominent position in the formation of these areas.

Over the past few years, the Brazilian livestock sector has undergone modifications by seeking technologies such as the improvement of forage plants, the replacement of nutrients in the soil, and the combat of pests, diseases, and invasive plants, consequently qualifying pasture management [3]. Such technologies enable to increase the forage yield of both seeds and dry biomass and also increase plant persistence in the field without the need to incorporate new areas for pasture formation [4]. The problem arising from breeding is usually that the selection aims to increase forage yield to the detriment of disease resistance.

Based on the development of technical knowledge of forage management, knowing the epidemiology and disease management is essential for the success of the sustainable exploitation of the crop in the field, as pathogenic species of *Bipolaris*, *Curvularia*, *Exserohilum*, *Fusarium*, *Phoma*, and fungi in stored seeds, such as *Aspergillus*, *Penicillium*, and *Rhizopus* have been reported to cause damage to the quality and establishment of forage plants [5-7]. Among these fungi, the species of *Bipolaris*, *Curvularia*, *Drechslera*, and *Exserohilum* represent a group of phytopathogens that severely attack forage grasses frequently, being known as dematiaceous hyphomycetes for their production of asexual spore pigments [8,9]. These phytopathogens are responsible for significant losses in plant yield. The symptoms caused by this group of fungi are generally leaf lesions caused by destruction of photosynthetic tissues, which can progress to necrosis and premature leaf death [10,11], limiting seed and forage formation.

It is known that, besides identifying the causal agent, quantifying the damage caused by these pathogens and diagnosing the period in which the plants are most susceptible to the disease are essential for the adoption of effective control measures, as these pathogens cause a reduction of dry biomass production and seeds with low sanitary quality. It was found that there are few studies on the severity and progress of diseases affecting forage grasses, especially on the ability of fungi to transmit from plants to the seeds produced. Knowledge of these aspects is very important to determine efficient control strategies.

Thus, the objective of this work was to study the severity and progress of leaf spot caused by *Exserohilum rostratum* inoculated in different phenological stages of *Brachiaria decumbens* cv. Basilisk and *Panicum maximum* cv. Mombasa, as well as to study the seed transmission of this pathogen.

Material and Methods

Seed sample and incidence of Exserohilum rostratum

Commercial samples of seeds of *B. decumbens* cv. Basilisk and *P. maximum* cv. Mombasa were obtained in the agricultural market, with both produced in the 2015/16 crop season. In order to check the primary incidence of *E. rostratum* in the seeds, the blotter test method [12] was used with 200 seeds per sample in four replicates of 50 seeds without prior disinfestation. The seeds were distributed in sterile plastic Gerbox boxes with two layers of filter paper sterilized and moistened with distilled water, and sterilized at a ratio of 2.5 times the mass of the dry substrate. The Gerbox boxes were placed in an incubation chamber under 12 h photoperiod and temperature of 25 ± 2°C for seven days [12]. After the incubation period, the analysis

of fungi present in the seeds was performed using a stereomicroscope, through which the morphological characteristics of the fungal structures were visualized. Genus-level identification was conducted based on specialized literature, such as Ellis [13], Barnett and Hunter [14], and Watanabe [15].

Plant obtaining, Exserohilum inoculation, and treatments

Seeds of the cultivars were sown in pots of 15 L capacity of substrate consisting of the mixture of sand and soil in the 1:1 ratio, with both being previously autoclaved for one hour at 120°C. The seeds were treated with imidacloprid (150 g a.i./L) + thiodicarb (450 g a.i./L) at a dose of 300 mL c.p./100 kg of seeds and metalaxyl-M (10 g a.i./L) + fludioxonil (25 g a.i./L) at a dose of 200 ml c.p./100 kg of seeds. Seed samples were packed in plastic bags, where the chemical treatment was conducted. The fungicide and insecticide was applied to the seeds, the plastic bags were inflated, and then seeds were stirred in order to allow better distribution of the product [16].

The pathogen was obtained from the sanitary analysis of *Brachiaria sp.* seeds, in which monosporic isolates were obtained and grown in PDA (potato-dextrose-agar) medium, and maintained in an incubation chamber under 12-h photoperiod and a temperature of 25 ± 2°C for seven days for inoculation reproduction. The isolates were subjected to molecular analysis for species identification. Molecular identification was conducted through DNA extraction [17], being subjected to polymerase chain reaction (PCR) followed by sequencing (Sanger). The identification was conducted by the Laboratory of Applied Molecular Biology of the Instituto Biológico - São Paulo, Brazil. After molecular analysis, the etiological agent that caused leaf spots in forage was identified and associated with the species *Exserohilum rostratum* (teleomorph *Setosphaeria rostrata*) (MT755902), with their respective fragments deposited on Gen Bank.

The experimental design was completely randomized with seven treatments in six replicates. After seedling emergence, the application of treatments was conducted using a manual sprinkler, with leaves being sprayed on their abaxial and adaxial faces, with suspension of conidia in the concentration of 1 x 10^6 conidia mL^{-1} of *E. rostratum*. The inoculation was conducted in six phenological stages of the plants, in which conidia suspension was applied only when the plant had reached the following pre-established moments: Vegetative I (30 days after emergence - DAE); Vegetative II (60 DAE); Booting I (Beginning of inflorescence development); Booting II (Full development of inflorescence); Reproductive I (green seeds); Reproductive II (seed maturation); and Control (plants without inoculation of the pathogen).

After inoculation, the plants remained in a dark humid chamber for 36 h and were then transferred to a natural environment to monitor the severity and progress of the disease. The tissues with symptoms were taken to the laboratory and subjected to superficial disinfestation to isolate the pathogens in PDA medium in order to confirm Koch's Postulates [18]. During the experimental period, the data of maximum and minimum temperature, relative air humidity, and precipitation were recorded to observe the influence of climatic conditions on the development of the pathogen in the plants. Climatic data were obtained from the Automatic Station located in the municipality of Gurupi, Tocantins, Brazil (11° 43' 45" latitude, 49° 04' 07" longitude, altitude of 278 m).

Evaluation of symptons of Exserohilum rostratum

After 48h of pathogen inoculation, evaluations of disease severity were conducted at two-day intervals. After five evaluations, the frequency was reduced, with seven-day intervals. Ten evaluations were conducted in total and the symptoms were evaluated using the grade scale described by Santos *et al.*, [19], in which the plant as a whole (all leaves) was considered, where: 0 - absence of symptoms; 1 - less than 1% of affected leaf area; 3 - between 1 and 5% of affected leaf area; 5 - between 6 and 25% of affected leaf area affected; 7 - between 26 and 50% of affected leaf area; 9 - more than 50% of affected leaf area. Subsequently, the severity scores were converted into percentages of diseased leaf area by the midpoint of each grade according to the number of evaluations for each trial, which was based on the onset of the disease. At the end of the evaluations, severity data were converted into an area under disease progress curve (AUDPC), according to the method described by Shaner and Finney [20].

Seed-plant transmission of Exserohilum rostratum

After inoculation of *E. rostratum* in the respective phenological stages Vegetative I and II, Booting I and II, and Reproductive I and II in both cultivars (previous subtopic), the completion of the plant cycle was awaited for seed harvesting. Plant seeds without inoculation were also collected, representing the control. A manual harvesting method was used in which all inflorescences were cut before the beginning of seed removal and placed on a lined and disinfected surface for drying. The seeds that came off the inflorescences were collected and taken to the laboratory for analysis.

Samples with 200 seeds of each treatment were submitted to the sanity test by the blotter test method [12]. The experimental design was completely randomized (CRD) with four replicates in a 7x2 factorial scheme, with seven treatments (phenological stage) of seeds submitted or not to disinfestation [12]. After the incubation period, a morphological analysis of the fungi present in the seeds was performed using a stereomicroscope in order to determine the existence of seeds infected with *E. rostratum* with the help of specialized literature, such as Watanabe [15].

Statistical analysis

Quantitative data (disease progression) were submitted to regression analysis and qualitative data were subjected to analysis of variance, with mean comparison by Tukey and Scott-Knott test at 5% probability. For the analysis, the SISVAR software was used [21].

Results

During conduction of the tests, which were exposed to the natural environment condition, little variation of the climatic characteristics was verified, except for the rains that occurred in all months, although with more voluminous precipitation between November and December, decreasing in the following months (Figure 1). This condition was favorable to the development of leaf spots. The average maximum and minimum temperatures of 32.2°C and 22.2°C, respectively, and relative humidity of 78.5% were also recorded.

Regarding the initial sanitary analysis of the seeds, before the seeds were treated for sowing in pots, there was no incidence of *Exserohilum sp.* Thus, it was ensured that the experiment was conducted free of natural infection caused by the fungus. The genera *Bipolaris*, *Cladosporium*, *Curvularia*, *Fusarium*, *Penicillium*, *Phoma*, *Phyllosticta*, *Pyrenochaeta*, and *Trichoderma* were found associated with the seeds of cultivars Basilisk and Mombasa (Table 1). However, it is worth mentioning, as aforementioned in the methodology, that

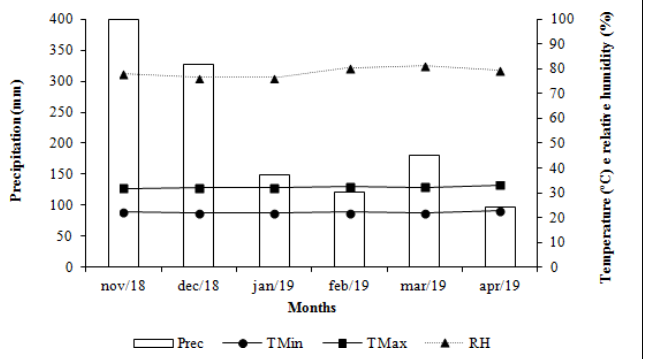

Figure 1: Climatic variables of precipitation (monthly accumulation - mm), maximum and minimum temperatures (°C), and average relative air humidity (%) observed during the test with forage plants in Gurupi, Tocantins, Brazil.

Table 1: Fungal incidence in Basilisk and Mombasa seed samples.

Fungal genus	Fungal incidence (%)	
	Brachiaria decumbens **cv. Basilisk**	***Panicum maximum*** **cv. Mombasa**
Bipolaris	31.0[1]	0
Cladosporium	3	18.5
Curvularia	11.5	4.5
Fusarium	4	7.5
Penicillium	0.5	0.5
Phoma	26.5	7
Phyllosticta	0	3.5
Pyrenochaeta	9.5	3
Trichoderma	1	0

[1]Mean of four replicates, with each replicate consisting of 50 seeds.

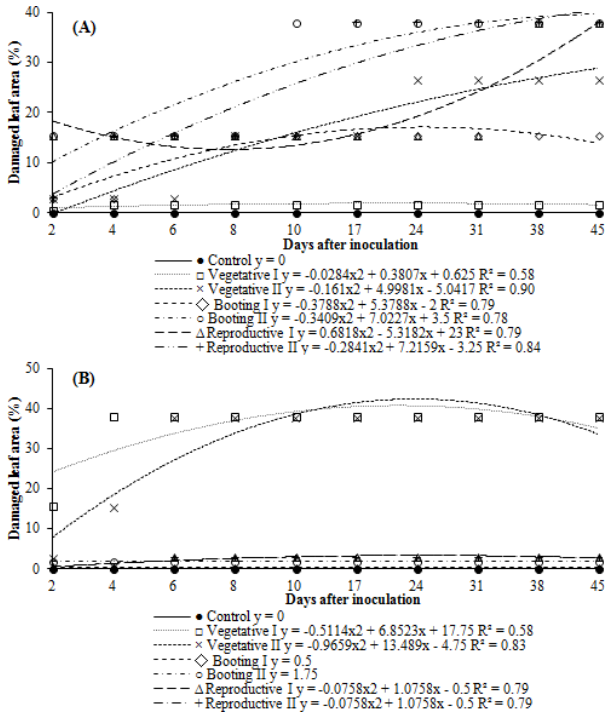

Figure 2: Temporal progress (% of damaged leaf area) of leaf spot caused by Exserohilum rostratum in Brachiaria decumbens cv. Basilisk (A) and Panicum maximum cv. Mombasa (B) after inoculation at different plant phenological stages.

the seeds were treated with fungicide and insecticide in order to obtain plants aimed to the study of the severity and progress of *E. rostratum*, preventing the emergence of other diseases.

The temporal progress of leaf spot caused by *E. rostratum* in *B. decumbens* cv. Basilisk and *P. maximum* cv. Mombasa was represented in Figure 2. In both forages, the initial symptoms of the disease appeared after two days of inoculation, regardless of the phenological stage in which it was applied. This indicates that the climatic conditions were favorable for pathogen infection. As expected, the plants used as controls remained healthy until the end of the cycle.

For Basilisk (Figure 2A), only the plants inoculated during the Vegetative I stage (30 DAE) presented affected leaf area below 2% over time. The other phenological stages presented an increase in disease severity until 45 days after the inoculation of *E. rostratum*, ranging between 15% and 38% of the affected leaf area. The plants with the greatest sensitivity to pathogen infection were inoculated at the Booting II and Reproductive I stages, presenting 15% of affected leaf area 48 hours after inoculation and progressing to 38% of affected leaf at 45 days after inoculation. Plants inoculated during the Reproductive II stage, despite finishing seed production, were also very sensitive to the fungus, with 15% severity at four days after inoculation, progressing to 38% at 17 days, and stabilizing the disease until the end of the evaluation. It is important to note that these forage grasses are of perennial cycle. Thus, they continue to till and emit new leaves after seed production, being still susceptible to the pathogen, and new infections may arise.

The cultivar Mombasa (Figure 2B) presented higher tolerance to *E. rostratum* severity in the Booting I and II and Reproductive I and II stages, in which it was infected by the pathogen (affected leaf area below 3%) but showed little or no disease progress until 45 days after inoculation. In Vegetative stages I (30 DAE) and II (60 DAE), the plants were more susceptible to the pathogen, with affected leaf area of 15% and 3%, respectively, at two days after inoculation, with progress after this period.

The disease severity, expressed by the AUDPC and considering inoculation at different phenological stages of *B. decumbens* cv. Basilisk and *P. maximum* cv. Mombasa is shown in Figures 3A and 3B, respectively.

It was observed that, for the cultivar Basilisk, when inoculation was conducted in the Vegetative stage II up to the Reproductive stage II, it resulted in greater disease progress in relation to the Vegetative stage I. The AUDPC of Vegetative stage I did not differ statistically from the plants used as control (Figure 3A).

When analyzing the AUDPC of the cultivar Mombasa, greater progress in disease severity was observed in Vegetative stages I and II. Although the stages of Booting I to Reproductive II did not differ statistically, it was considered that Booting I and II were significantly equal to the control (Figure 3B).

Symptoms caused by *E. rostratum* in *B. decumbens* cv. Basilisk leaves started with small leaf spots of reddish-brown color (Figure 4A and 4B), resulting in coalescence, drying, and progressive leaf necrosis (Figure 4A). The characteristic symptoms of *E. rostratum* are

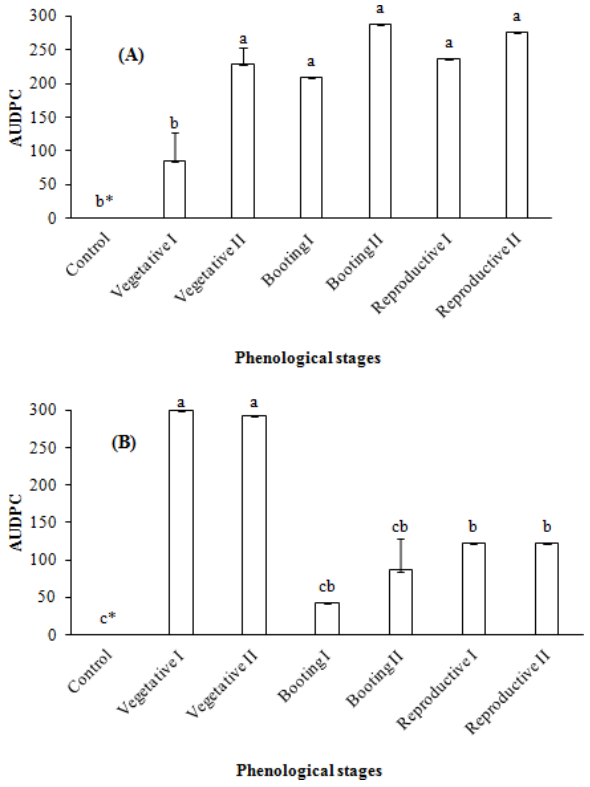

Figure 3: Area under disease progress curve (AUDPC) for leaf spot caused by Exserohilum rostratum at different phenological stages of Brachiaria decumbens cv. Basilisk (A) and Panicum maximum cv. Mombasa (B). (*Same letters above each column do not differ by Tukey's test at 5% probability.)

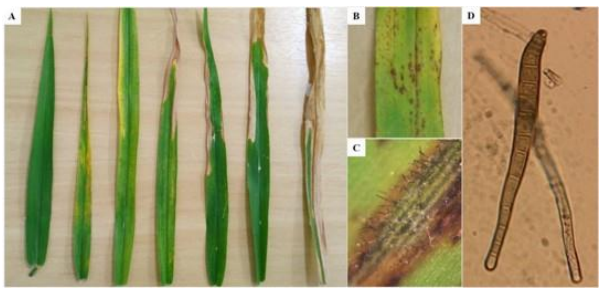

Figure 4: Progress of leaf spot caused by *Exserohilum rostratum* in *Brachiaria decumbens* cv. Basilisk: Evolutionary symptoms of the disease with coalescence of lesions followed by necrosis and drying of the limbus (A); Initial symptoms of the disease (B); Signs of the pathogen in the leaf tissue (C); Fungal conidium (D). Photo: Own authorship.

elongated, elliptical lesions that are gray or brown colored and varying in length, occurring initially in older leaves. Figures 4C and 4D show the signs of the pathogen after incubation of the leaf tissue infected, with the presence of reproductive structures (conidia). In the field, under favorable conditions to the development of the pathogen, this sporulation usually occurs (Figure 4C) and conidia dispersal occurs by wind or rain/irrigation water, spreading the pathogen to other areas.

The main means of survival, dissemination, and introduction of pathogens in new areas of cultivation is through seeds, which come mostly from sick plants. In this sense, for each treatment, seeds were collected to check the phenological stage in which seed-plant transmission was observed (Table 2).

Table 2: Incidence of *Exserohilum rostratum* in seeds produced by plants inoculated at different times (phenological stages).

Phenological stage of inoculation	B. *decumbens* cv. Basilisk		P. *maximum* cv. Mombasa	
	WD[1]	DS	WD	DS
Control	0.0 dA[2]	0.0 bA	0.0 dA	0.0 cA
Vegetative I	0.0 dA	0.0 bA	1.0 dA	0.0 cA
Vegetative II	12.0 cA	1.0 bB	2.0 dA	0.0 cA
Booting I	59.0 aA	4.0 bB	31.0 cA	12.0 bB
Booting II	51.0 aA	7.0 bB	30.0 cA	10.0 bB
Reproductive I	61.0 aA	27.0 aB	86.0 aA	48.0 aB
Reproductive II	37.0 bA	12.0 bB	67.0 bA	53.0 aB
CV %	38.16		23.98	

[1]WD-Without seed disinfestation; DS - With seed disinfestation. [2]Means of four repetitions. Means followed by the same lowercase letter in the column and uppercase letter in the line do not differ by Scott-Knott test at 5% probability.

Basilisk plants inoculated in the Vegetative II, Booting I and II, and Reproductive I and II stages produced seeds infected with *E. rostratum*, with those being the periods in which the greatest disease severity was observed. The seeds were analyzed with and without superficial disinfestation, a technique that allows the elimination of contaminating microorganisms present on the outer shell of a seed (tegument). The seeds from plants inoculated in the phenological stages mentioned above, without disinfestation, presented statistically higher incidence of *E. rostratum* than disinfected seeds. However, even in a lower incidence, the possibility of pathogen transmission was not discarded, with emphasis on the seeds in which *E. rostratum* was inoculated during the Reproductive II stage, which had 27% transmission (Table 2).

The cultivar Mombasa, which presented higher disease severity during the Vegetative I and II stages when inoculated, produced seeds originating from these inoculation stages with the lowest percentages and incidence of the fungus, with or without superficial disinfestation. The highest seed-plant transmission rates were observed during the Reproductive I and II stages (86% and 67% - WD) and with statistically lower transmission rates for disinfected seeds (48% and 53% - DS) (Table two).

Discussion

Leaf spots caused by dematiaceous fungal infections can cause large losses in agricultural activity [22-25] as a result of the decrease or total destruction of the plant photosynthetic area. Conditions such as a susceptible host, a virulent pathogen, and a favorable environment are the key factors for infection and for the development of these diseases to occur [26,27].

As observed, under average temperature of 27°C, relative air humidity of approximately 80%, and frequent precipitation, the forages *B. decumbens* cv. Basilisk and *P. maximum* cv. Mombasa were susceptible to *E. rostratum*, although the disease severity ranged according to the phenological stages in which the pathogen was inoculated. According to the Köppen classification, the climate of the study region is Aw, defined as hot and humid tropical with a rainy season in summer [28], resulting in favorable weather conditions for fungal infection during the conduction of the experiment, as there

was predominance of high temperatures and high relative humidity.

Regarding disease severity and production of infected seeds, areas where the disease occurs more severely do not always favor high levels of seed infection. On the other hand, in areas where the disease is manifested with less intensity, high levels of seed infection can be detected, which are more associated with the stage of crop development, making it more susceptible or not to seed infection [29-31].

In this study, the highest severity of *E. rostratum* in the cultivar Basilisk was observed from the Vegetative II to Reproductive II stages, coinciding with higher levels of seed transmission and infection. On the other hand, the highest severity of *E. rostratum* in the cultivar Mombasa was observed during Vegetative stages I and II, and the highest seed infection from the Booting I stage. Thus, it was found that the most susceptible period of these forages to the pathogen did not necessarily coincide with the period of greatest susceptibility to seed infection. Vechiato *et al.*, [32], who analyzed seeds of the species *B. decumbens*, *B. brizantha*, and *P. maximum*, reported a significant incidence of *E. rostratum*, although they did not test the disease pathogenicity and severity.

For the cultivar Basilisk with inoculation of *E. rostratum* from the Booting II until Reproductive II stages and for the cultivar Mombasa with inoculation in the Vegetative I and II stages, leaf spot severity was verified in the first four days, with relatively high values of affected leaf area (between 15% to 38%). The multiple lesions that occur in the plant leaf tissue, when in a favorable environment, develop very quickly, suggesting the action of fungal toxins involved in the infection process [33], corroborating with reports of the presence of a nonspecific toxin, monocerine, in the water-soluble phytotoxic compounds of *E. turcicum* [34,35] and *E. rostratum* [36]. Robeson and Strobel [37] isolated the monocerin of *E. turcicum* and reported that the compound demonstrated phytotoxic activity on the growth of *Sorghum halepense* and *Cucumis sativus*. However, more works relating the role that the monocerin present in *Exserohilum* sp. plays in the process of plant infection are needed.

Sick plants tend to produce seeds infected by the pathogen. This fact was demonstrated in the present study and also by other authors [31,38]. Thus, it can be inferred that among the consequences resulting from the plant-seed transmission of fungi, it is possible to mention the spread of pathogens by the seeds to exempt areas [7,39] and the reduction of the physiological quality of seeds, which may reduce germination and emergence [40-42]. In addition, when contaminated seeds are aimed to consumption, such as grains, they can present harmful toxins to animals and humans [43,44].

According to Neergaard [29], the occurrence of a disease whose causal agent comes from the seed depends on the three following main factors: inoculum quantity in the seeds, transmission rate, and its rate of progress in the field. Picinini and Fernandes [45] reported that lots of barley seeds presenting infection levels above 5% of *Bipolaris sorokiniana* (dematiaceous) were sufficient to produce the inoculum necessary for the development of an epidemic in the field under favorable conditions. Assuming this condition in this study, in which *Brachiaria* and *Panicum* seeds presented infection of 27% and 53%, respectively, their use for the formation of new pasture areas would result in a large production of inoculum and infection of the plants with *E. rostratum*.

The incidence of *E. rostratum* decreases seed quality in several aspects regarding sanitary and visual quality and their production, whether by forage plants or other crops of agricultural importance [46]. According to Silva *et al.*, [42], seeds infected with *E. rostratum* germinate less due to the colonization of the inner seed tissues by the pathogen, such as the pericarp, endosperm, or even the embryo. It is likely that *E. rostratum* infects the inner seed tissues because even after disinfestation its detection associated with the seeds is possible [47].

In this study, *E. rostratum* transmission was observed. In addition, even after the disinfestation of *Brachiaria* and *Panicum* seeds, incidence of the fungus in the seeds was observed. Reis and Casa [48] discussed the association of *B. sorokiniana* with barley seeds, in which the fungus located internally in the seed in the form of mycelium in the pericarp and endosperm would result in higher transmission rates, as the pathogen has higher efficiency to survive and subsequently move to the root and shoot organs of plants. Thus, *B. decumbens* cv. Basilisk and *P. maximum* cv. Mombasa seeds produced and evaluated in this study could be potential vectors of dissemination and transmission of *E. rostratum.*

E. rostratum has also been reported to cause severe leaf spots in other grasses such as maize and rice [42,49] and even in other cultivated plants, such as açaí, coconut, beans, and fishtail palm [50,51]. In addition, forage grass species have been very susceptible to dematiaceous fungi that cause leaf spots, such as *Bipolaris* sp., *Curvularia* sp., and *Helmintosporium* sp., among others [7,11,52], which demonstrates the need for the development of resistant cultivars and/or phytosanitary products for the adequate management of the disease. Most grass-infecting dematiaceous hyphomycetes have similar disease cycles [8], initiating infection by older leaves by conidia produced from infected tissues or crop residues, with repeated cycles [53,54] and in severe cases, plant weakening, with loss of vigor and emergence.

After analyzing the temporal progress of the disease caused by *E. rostratum* and seed-plant transmission in the forage cultivars Basilisk and Mombasa, the need to establish control measures was emphasized mainly in fields of seed production, in which there is *E. rostratum* incidence, especially considering the phenological stages of the plants most susceptible to the disease. Therefore, the production of forage seeds with sanitary quality control is essential for the success of the seed production sector in Brazil.

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