



## Effect of biological treatments and water deficit on *Macrophomina phaseolina* infection in sorghum

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### ABSTRACT

**Background/Objective.** Tamaulipas is the main producer of sorghum (*Sorghum bicolor*) in Mexico, but its unit yields are affected by biotic and abiotic factors, where *Macrophomina phaseolina* stands out, whose control is partially effective. In this work, the effect of biological treatments on the development of *M. phaseolina* in sorghum seedlings grown in a greenhouse in Reynosa, Tamaulipas, was evaluated.

**Materials and Methods.** Two experiments were established under a factorial arrangement in plots subdivided 2 x 2 x 5 where the large plots were the Pioneer 8282 and RB Patrón hybrids; the medium plots the levels of humidity, irrigation and water deficit; and the small plots the biological treatments: strain T808 of *Trichoderma koningiopsis*, strain AF36 of *Aspergillus flavus*; Kuali® mycotoxin chelating agent; control inoculated with *M. phaseolina* and absolute control.

**Results.** The RB-Patrón hybrid showed the greatest damage to *M. phaseolina* and the lowest growth (plant height: 10%) and accumulation of dry biomass (between 35-40%). Water deficit increased charcoal rot infection and reduced sorghum growth. *T. koningiopsis* strain T808 reduced the severity of charcoal rot (value of 4.6) while the other biological treatments exhibited severity values greater than 6.0.

**Conclusion.** The treatments with better sorghum growth and less damage by *M. phaseolina* (T808, P-8282) should be evaluated under field conditions to confirm the viability of their application in commercial sorghum production in northern Tamaulipas.

**Keywords:** *Sorghum bicolor*, *Aspergillus flavus*, *Trichoderma koningiopsis*, Biological control, Charcoal rot



## INTRODUCTION

Tamaulipas is the main producer of sorghum in Mexico, accounting for 60% of the country's total. In 2023, a total of 806,000 were planted, with a production of 2.3 million tons and a total yield of 2.8 t ha<sup>-1</sup>. The productivity of the crop is low, since 73% of the sorghum in Tamaulipas is grown under rainfed conditions, with average yields of 2.6 t ha<sup>-1</sup>, far from the sorghum yield (over 6 t ha<sup>-1</sup>) grown with irrigation in other regions in Mexico (SIAP, 2024). Under the rainfed conditions of Tamaulipas, there are frequent and intermittent conditions of drought and high temperatures, which affect the growth and development of the crop, as well as the yield and quality of the production. Environmental stress increases the incidence and damage by diseases and pests (Díaz-Franco y Montes-García, 2008).

One of the most important diseases in sorghum in Tamaulipas is the charcoal rot in the stem, the main causal agent of which is the fungus *Macrophomina phaseolina*. The fungus mainly infects the stems and roots of the plant and causes the typical "charcoal rot" or "ashy blight" due to the production of microsclerotia and mycelia (Little *et al.*, 2023). The predominance of the disease and the favorable conditions for its development have produced interest in the production of sorghum germplasm that is resistant both to environmental stress (drought and high temperatures) and to charcoal rot, with the aim of increasing production in the region (Williams-Alanís *et al.*, 2009; 2021; Montes-García *et al.*, 2020; 2021). Considering that the highest proportion of the surface dedicated to sorghum is planted under rainfed conditions and that inputs used for their production under these conditions are scarce (Alejandro-Allende *et al.*, 2020), it is convenient to develop, evaluate and propose economic alternatives that are not contaminant and are effective in the control of diseases and crop pests.

Biocontrol strategies for diseases as management options are available, so if they offer favorable results, they could extrapolate to the commercial sorghum production of Tamaulipas (Ángeles-Núñez *et al.*, 2018). The options that have been considered in the case of *M. phaseolina* are diverse (Márquez *et al.*, 2021; Rajput *et al.*, 2023). One of them, perhaps the most widely used in sorghum and other crops, are the fungi of the *Trichoderma* spp. genus, which use varied mechanisms such as antibiosis, mycoparasitism, competition, promotion of plant growth, improvement of the tolerance of the host to the abiotic stress and the activation of the defense system against pathogens such as *M. phaseolina*. Additionally, they produce volatile and non-volatile compounds and/or siderophores to develop their biocontrol (Shahriar *et al.*, 2022, Rubayet and Bhuiyan, 2023; Rajpu *et al.*, 2023). Martínez-Salgado *et al.* (2021) reported that *T. koningiopsis* (T-K11) displayed the highest growth and inhibition rates for the growth of *M. phaseolina* (71%) *in vitro*, as well as the highest production of peanut (*Arachis hypogaea*) (1.60±0.01 t ha<sup>-1</sup>) and less damage caused by charcoal rot in the field. Arispe-Vázquez *et al.* (2024) observed the highest inhibition of the growth of *M. pseudophaseolina* due to *T. reesei* strains in chili plants grown in the winter.

Another option, whose application in the management of *M. phaseolina* has not been reported, is the fungus *Aspergillus flavus*. Despite being well-known that the fungus produced aflatoxins with a carcinogenic potential in humans and affects the quality and productivity of several crops, isolates are reported, on the other hand, which do not produce aflatoxins (atoxigenics) naturally and that, in addition, compete for space and nutrients with the aflatoxin-producing isolates during the development of the crops,

reducing contamination by aflatoxins. *A. flavus* strain AF36 is a biological control strategy by competitive exclusion of aflatoxigenic fungi in cotton plants (Cotty and Bayman, 1993). King *et al.* (2011) and Rangel-Muñoz *et al.* (2022) point out that AF36 controls infections caused by toxigenic fungi in cotton plants (*Gossypium hirsutum*), maize (*Zea mays*) or peanut. Also, in fruit trees such as pistachio (*Pistacia vera*) and Spanish almond (*Prunus dulcis*), *A. flavus* strain AF36 significantly reduces contamination from aflatoxins (García-López *et al.*, 2018, 2024).

Finally, there is the option of detoxifying chemical agents that suppress or reduce adsorption, increase excretion or modify the mode of action of substances such as mycotoxins, since these bind to the compound through chemical adsorption. An outstanding case is that of calcium and sodium aluminosilicates (HSCAS), used successfully in some crops (Habschied *et al.*, 2021; Hassan and Afzal, 2022; Aloui *et al.*, 2023). Applying sodium silicates on the leaves or soil reduces infections by *M. phaseolina* in soybean (Rajput *et al.*, 2023). Neither are there earlier reports on the use of HSCAS in sorghum for the management of *M. phaseolina*. The aim of this study was to evaluate the effect of biological treatments (*T. koningiopsis* fungus, strain T808 and *A. flavus* strain AF36; and the chelating agent calcium and potassium aluminosilicate, Quali®) on the development of *M. phaseolina* in sorghum grown in a greenhouse, under conditions of water stress.

**Sorghum hybrids.** The study included two sorghum hybrids Pioneer® P-8282 and B-Patrón® of the National Forestry, Agriculture and Livestock Research Institute (INIFAP). P-8282 stands out for its adaptation to northeastern Mexico, its tolerance to lodging and to drought (Pioneer-Dupont, 2017); RB-Patrón is tolerant to high temperatures and drought, as well as to infection by *M. phaseolina* in northern Tamaulipas (Williams-Alanís *et al.*, 2004).

**Biological treatments.** Three biocontrol strategies were evaluated in the study: 1) la cepa AF36 de *A. flavus* (Syngenta®; Arizona, EUA); 2) *T. koningiopsis* strain T808 (Hernández-Mendoza *et al.*, 2011) and 3) the aflatoxin-chelating agent Quali (Azul Natural®; Durango, Mexico. <https://azulnatural.net/products/>), based on calcium and potassium aluminosilicate. In all cases, the method of application and the doses used were based on the instructions by the manufacturers. Both T808 and AF36 were inoculated during the plantation of sorghum, whereas Quali was applied to the foliage of the plants 15 days after planting (dap) (Quali solution/sterile mineral oil 5:1 p/v).

**Inoculation of *M. phaseolina*.** The *M. phaseolina* inoculant was prepared from strain HMP5, isolated from soybean (*Glycine max*) in Altamira, Mexico and with high virulence in bean plants (*Phaseolus vulgaris*), sorghum (*Sorghum bicolor*) and soybean (*Glycine max*) (Mayek-Pérez *et al.*, 2002). The strain was grown on Potato-Dextrose-Agar (PDA) medium at 30 °C for six days in the dark. The microsclerotia and mycelia were ‘scraped’ off the fully colonized dishes and a solution was prepared on sterile deionized water. The corresponding treatments were provided with a concentration of 10<sup>5</sup> culture forming units per experimental unit during the plantation of sorghum, along with the seeds (De la Peña-Devesa *et al.*, 2009).

**Levels of humidity.** The treatments were planted under two humidity conditions: irrigation and drought. Under irrigation, humidity was maintained at field capacity from planting until the experiments concluded; under drought conditions, irrigation was

suspended 25 dap until permanent wilting was reached. The plants were kept in this condition for three days and a recovery irrigation was applied at field capacity. In the rest of the experiments, the plants were kept under normal irrigation conditions (Mayek-Pérez *et al.*, 2002).

The sorghum hybrid seeds were disinfested in a 1% sodium hypochlorite solution for. The seeds were rinsed with sterile deionized water and dried using sterilized paper and then planted in 36-cell plastic trays containing sterile substrate (Topsoil®; Marisville, USA), with six seeds per cell. The 20 treatments evaluated resulted from the combination of the factors and levels considered: two sorghum hybrids x two humidity levels (irrigation, drought) x 5 inoculation treatments (three for biocontrol: T808, AF35, Quali plus two controls: the hybrids without inoculation with no control agent and the hybrids inoculated with HMP5). The treatments displayed a factorial arrangement in subdivided plots and were randomized in a completely randomized experimental design with six repetitions. The large plots corresponded to sorghum hybrids, large plots to humidity levels and small ones, to the biocontrol treatments. The experimental unit was one cell with six seeds. The experiment was carried out twice.

**Plant height.** The variable was measured 10, 16, 22, 28, 34 and 40 dap in three plants of each experimental unit; a ruler was used to measure height from the base of the plants in the soil to the apex of the leaves.

**Severity of charcoal rot.** On the same dates of the plant height evaluation, the severity of the charcoal rot on the plants of an experimental unit per treatment was quantified. The plants were previously washed with deionized with sterile deionized water. The severity was evaluated using the scale by Abawi and Pastor-Corrales (1990), which includes nine values (from 1 to 9, where 1=no visible symptoms and 9=75% or more of the tissues with lesions, decaying of the root and extensive growth of the fungus).

**Dry weight of biomass.** The plants on which the severity of *M. phaseolina* was evaluated were used to estimate the dry weight of the root, the aerial part and total dry weight. The plants were placed in paper envelope and dried in a forced-air oven at 60 °C for five days (FX5, Shel-Lab; Cornelius, OR, USA). Subsequently, the dry weight was determined using an analytical scale (BP 2100S, Sartorius; Göttingen, Germany).

The data of the measured variables were placed under an analysis of variance (ANOVA). In the cases in which significant differences were found, Tukey's honest significant difference (HSD,  $p=0.05$ ) values were calculated for the comparison of means between treatments (Martínez-Garza, 1988). The statistical analysis was carried out using SAS 9.0 for Windows (SAS Institute; Cary, NC, USA; [https://www.sas.com/es\\_mx/home.html](https://www.sas.com/es_mx/home.html)).

**Experiment 1.** The ANOVA found significant differences ( $p<0.05$ ) between inoculation levels for all the variables measured; among hybrids and humidity levels only for plant height in the first evaluation, whereas for the sixth evaluation significant differences ( $p<0.05$ ) were found between hybrids, humidity levels and inoculation levels for the severity of rot charcoal; among hybrids for plant height and the dry weight of the stem; and between moisture levels for the dry weight of stem and roots. Simple and triple interactions displayed no significant differences ( $p>0.05$ ) in the first evaluation for any variable, but significant differences were found in all the variables measured for most interactions in the sixth evaluation (data not included).

In the first evaluation hybrid P-8282 presented a greater plant height (5.6 cm), whereas the lowest plant height was found under irrigation conditions (5.1 cm). Among biological treatments, the absolute control recorded no damage from *M. phaseolina* (1.0) and also presented the greatest plant height (5.9 cm). Applying T808 presented the lowest dry weight for stem (16 mg), whereas the treatment with *M. phaseolina* presented the greatest dry weight for root (30 mg). For the sixth evaluation, RB-Patrón presented the greatest damage from charcoal rot (6.0), which translated into lower plant growth (lower plant height averages, dry weight in stem and dry weight in root). Drought increased the damage from charcoal rot and also reduced sorghum growth regarding plant height and dry weight in stem and root. In turn, strain T808 reduced the severity of charcoal rot to 4.6, while the biological treatments displayed severity values of over 6.0 (Table 1).

The triple interaction (hybrids x humidity x biological treatments) displayed a wide variation of responses, since the severity of *M. phaseolina* went from a value of 1 to 9, hence accumulation of total dry biomass accumulation, since the values were not greater than 200 mg per experimental unit. The biological treatments with the least damages per charcoal unit were Kualí in RB-Patrón under irrigation and T808 in RB-Patrón under drought (Figure 1A).

**Experiment 2.** The ANOVA found significant differences ( $p < 0.05$ ) between hybrids for the variables severity of *M. phaseolina*, plant height and dry weight of stem (first evaluation) for the dry weights of stem and root (sixth evaluation). There were significant differences ( $p < 0.05$ ) between levels of humidity only for the severity of charcoal rot (first evaluation), whereas in the sixth evaluation, differences ( $p < 0.05$ ) were found in all the variables measured. Significant differences ( $p < 0.05$ ) were found for the severity of charcoal rot in both evaluation dates and for plant height only in the first measurement of the variable. The simple and triple interactions displayed no significant differences ( $p > 0.05$ ) in the first evaluation of any of the variables, yet significant differences ( $p < 0.05$ ) were found in all the variables measured for all interactions in the sixth measurement of variables (data not included).

The RB-Patrón hybrid displayed greater severity of damage (1.4) in comparison with P-8282, which displayed a value of 1. However, it also displayed the highest averages in plant height and dry weight for stem and root in the first measurement and the lowest dry weight for stem and root in the sixth evaluation. The greatest damages due to charcoal rot were observed in plants under drought in comparison with plants under irrigation: 1.4 vs 1.0 in the first measurement and 2.8 vs 2.0 in the second one, respectively. Drought conditions reduced the plant height by 10% and the accumulation of biomass at the end of the experiment between 25 and 40%. In the first measurement, the control presented no damage from *M. phaseolina*, but it did present the greatest plant height. By contrast, in the sixth evaluation, the lowest level of damage caused by charcoal rot were found in T808 (2.2), while the other biological treatments displayed severity values greater than 2.7 (Table 2). The triple interaction (hybrids x humidity x biological treatments) displayed a lower variation in its response to *M. phaseolina*, with severity values of 1 to 4. Due to this, the accumulation of total dry biomass was higher than that observed in experiment 1 (values of up to 1800 mg per experimental unit). The biological treatments with the least damage from charcoal treatment were Kualí, AF36 and T808 in hybrid P-8282 under drought, as well as *T. koningiopsis* strain T808 in RB-Patrón under irrigation (Figure 1B).

**Table 1.** Means comparison of the main effects for the severity of damage by *M. phaseolina*, plant height and accumulation of biomass in sorghum. First and sixth evaluations in experiment 1.

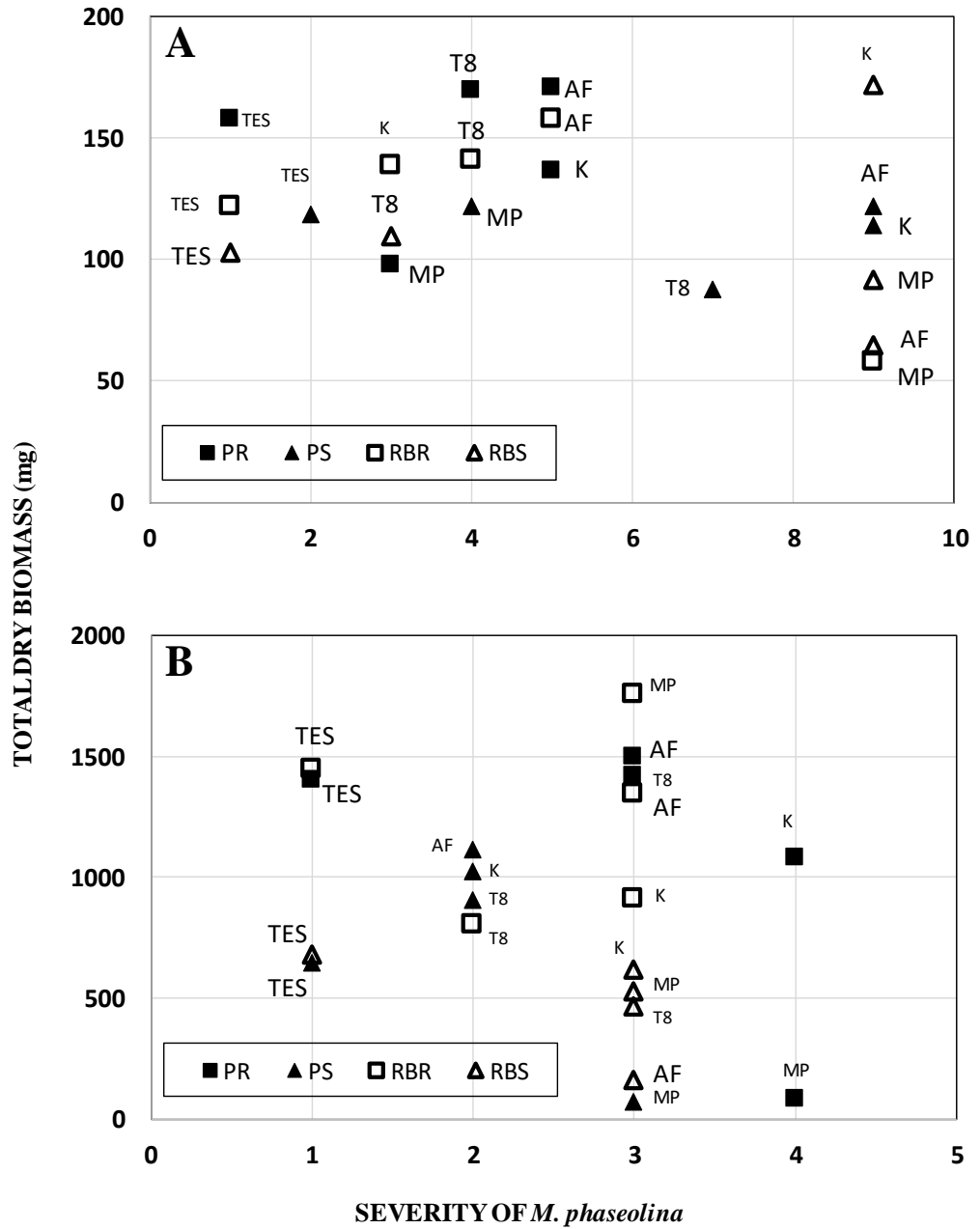
Treatment	First evaluation (10 das)				Sixth evaluation (40 das)			
	Disease severity	Plant height (cm)	Aerial dry weight (mg)	Root dry weight (mg)	Disease severity	Plant height (cm)	Aerial dry weight (mg)	Root dry weight (mg)
Hybrids	1.0	5.6	19	18	4.6	7.3	78	60
Pioneer 8282	1.3	5.1	20	20	6.0	6.8	66	54
RB-Patrón	0.3NS	0.3**	2NS	2NS	1.3**	0.4**	8**	11NS
H LSD								
Humidity								
Irrigated	1.0	5.1	18	20	2.6	7.2	84	63
Water deficit	1.3	5.6	20	20	8.0	6.9	59	50
H LSD	0.3NS	0.3**	2NS	2NS	1.3**	0.4NS	8**	11**
Inoculation								
<i>M. phaseolina</i>	1.0	4.9	22	30	6.9	6.9	62	50
AF36	2.1	5.3	21	2	6.7	7.1	82	56
T808	1.2	4.8	16	19	4.2	6.7	68	58
Kuali	1.0	5.5	20	18	7.9	7.1	71	67
Control	1.0	5.9	19	15	1	7.4	70	51
H LSD	0.6**	0.7**	4**	4**	2.6**	0.8NS	18NS	25 NS

**Table 2.** Means comparison of the main effects for the severity of damage by *M. phaseolina*, plant height and accumulation of biomass in sorghum. First and sixth evaluations in experiment 2.

Treatment	First evaluation (10 das)				Sixth evaluation (40 das)			
	Disease severity	Plant height (cm)	Aerial dry weight (mg)	Root dry weight (mg)	Disease severity	Plant height (cm)	Aerial dry weight (mg)	Root dry weight (mg)
Hybrids	1.0	10.6	105	14	2.3	17.3	720	360
Pioneer 8282	1.4	11.4	88	15	2.5	17.2	586	223
RB-Patrón	0.3**	0.3**	10**	2NS	0.4NS	0.6NS	77**	43**
H LSD								
Humidity								
Irrigated	1.0	11.1	103	15	2.0	18.1	801	367
Water deficit	1.4	10.9	90	13	2.8	16.5	513	221
H LSD	0.3**	0.3NS	10NS	2NS	0.4**	0.6**	77**	43**
Inoculation								
<i>M. phaseolina</i>	1.4	10.9	99	15	3.0	16.5	550	313
AF36	1.8	10.6	92	13	2.7	17.7	742	315
T808	1.6	10.7	88	15	2.2	16.8	634	238
Kuali	1.2	11.1	105	15	2.8	16.7	629	288
Control	1.0	11.6	97	15	1	18.0	700	335
H LSD	0.4**	0.7**	25NS	4NS	0.7**	1.6NS	195NS	109NS

Both experiments showed that the greatest damage caused by charcoal rot took place in the hybrid RB-Patrón, particularly when grown under conditions of drought. This led to the lowest accumulation of dry biomass. Both stress conditions, alone or combined (fungus/drought), affect growth and the accumulation of dry biomass in sorghum hybrids (Perumal *et al.*, 2020; Marquez *et al.*, 2021; Little *et al.*, 2023), particularly during the vegetative stage of the development of the species, which was the period in which this

study was performed. Drought reduces the growth of the aerial section and of the roots of sorghum, as well as the accumulation of dry biomass due to the reduction of the extensibility of the wall and cell turgidity (Queiroz *et al.* 2019; Abreha *et al.*, 2022).



**Figure 1.** Relationship between the severity of charcoal rot and the total dry biomass accumulated in the sixth evaluation in sorghum grown in a greenhouse. (A) Experiment 1, (B) Experiment 2. PR=Pioneer 8282/Irrigation; PS=Pioneer 8282/Water deficit; RBR=RB Pattern/Irrigation; RBS=RB Pattern/Water.

In general, the treatment with *T. koningiopsis* strain 808 had the best control results for *M. phaseolina* in terms of the least damage from charcoal rot and accumulation of dry biomass. The combinations of Kuali with RB-Patrón under irrigation and T808 with RB-Patrón under drought, although with the accumulation of intermediate biomass (experiment 1), also provided promising results, whereas in experiment 2, the best

biocontrol responses were obtained with Kualí, AF36 or T808 combined with P-8282 under water deficit and T808 with RB-Patrón under irrigation. Additionally, certain specific combinations of biocontrol treatments (*T. koningiopsis*, *A. flavus* atoxigénica, Kualí) displayed percentages in the reduction of severity of charcoal rot in sorghum, as well as the reduction of the effects on the growth and accumulation of dry biomass.

Reports of the effect of *T. harzianum*, *T. viride*, *T. asperellum* or *T. koningiopsis* strains on the *in vitro* suppression of growth and of the damage caused by several phytopathogenic fungi, including *M. phaseolina*, as well as in diverse crops including sorghum have been constant and with similar results (Yassin *et al.*, 2021). Isolations of the *Trichoderma* genus and specifically *T. koningiopsis* strains have shown positive effects on sorghum, both improving its response to abiotic stress and in the defense against infection by pathogens such as *M. phaseolina* (Bhutada and Shinde, 2023; Kubiak *et al.*, 2023; Rubayet and Bhuiyan, 2023; Rajpu *et al.*, 2023). For example, in peanut grown in Puebla, Mexico, Martínez-Salgado *et al.* (2021) reported that *T. koningiopsis* presented the highest growth rate and the highest *in vitro* inhibition of *M. phaseolina* growth (71%), as well as the highest peanut production ( $1.60 \pm 0.01$  t/ha<sup>-1</sup>) and the least damage from charcoal rot in the field. Ruangwong *et al.* (2021) pointed out that *T. koningiopsis* contains azetidine, 2-phenylethanol and ethyl-hexadecanoate, compounds associated to antibiosis and the suppression of the mycelial growth of *M. phaseolina* reported by Martínez-Salgado *et al.* (2021).

Although authors such as Quiroz *et al.* (2008) observed that *Aspergillus* sp. strains could become an option in the biological control of fungi such as *M. phaseolina*, they did not recommend its application in the field since its capacity to produce aflatoxins and other mycotoxins in the species of the genus is well-known. However, the results by Almeyda-León *et al.* (2020) are promising, since after 312 isolates obtained from planted and unplanted soils, they identified 212 fungal morphotypes, three of which inhibited the growth of *M. phaseolina* by over 60%. Two of those morphotypes were identified as *Aspergillus* sp. and *Penicillium* sp. The results showed that fungal strains with potential antagonistic activity against *M. phaseolina* occur naturally. Therefore, the fungi of the *Aspergillus* genus native to Mexican planted and unplanted soils are potentially apt for applying in biological *M. phaseolina* control in sorghum; however, it is necessary to ensure the atoxigenic condition of the strains with the potential to inhibit the development of charcoal rot in sorghum grown in northeastern Mexico.

In the case of the chelating agent made from calcium and potassium aluminosilicate (HSCAS), although to a lesser extent, it also presented results that suggest the need for later evaluations in the field to help define whether it has possibilities of being used in the commercial production of sorghum in northern Tamaulipas. Aluminosilicates have been successfully evaluated and applied in the management of aflatoxicosis derived from the fungus *A. flavus*, particularly in the industry of balanced feeds that use sorghum and maize grains that, if contaminated with aflatoxins, would be an important health problem for livestock and later for the consumer of products derived from that livestock, such as meat and milk (Peng *et al.*, 2018).

The next step is to extend those evaluations to the complete biological cycle of the crop in various experimental sites in northern Tamaulipas and verify the observations under controlled conditions. The results are promising in that sense, since this is the first report of results indicating that, both the *A. flavus* strain AF36 and the chelating agent

Kuali display positive effects on the biocontrol of *M. phaseolina* on the pathosystem that integrates with sorghum under rainfed conditions.

The short-term challenge is to define the mode of action of the antagonistic microorganisms or of the chelating agent in the case of *M. phaseolina* in sorghum, which will help advance in the development of potential biopesticides based on the establishment of the interactions in dynamic systems (biocontrol agent-phytopathogen, biocontrol agent -plant or biocontrol agent-plant-phytopathogen). In addition to verifying the antagonistic and phytosanitary properties of the fungus, its toxicity in humans and the environment must be evaluated. It is also important to pay attention to the treatment or application methods on the soil, plants or seeds, as well as the formulation and the large-scale fermentation of the biopesticides. A limitation is to effectively transfer the results from the laboratory to the field, although the most important challenge to developing biological products is to raise public awareness on the efficiency and safety of bioproducts based on *Trichoderma* sp. (Navi and Yang, 2020; Kubiak *et al.*, 2023; Kumar *et al.*, 2023).

The RB-Patrón hybrid displayed the greatest damage from charcoal rot in comparison with P-8282, as well as the least growth (plant height: 10%) and accumulation of dry biomass (between 35 and 40%). Drought increases the severity of charcoal rot and reduces the growth of sorghum. *T. koningiopsis* strain T808 reduced the severity of the charcoal rot (value of 4.6), whereas the other biological treatments presented severity values greater than 6.0.

The triple interaction (hybrids x humidity x biological treatments) displayed variable results, since the biological treatments with the least damages caused by charcoal rot were Kuali, AF36 and T808 in hybrid P-8282 under drought, as well as T808 in RB-Patrón under irrigation. These treatments should be evaluated under field conditions to confirm the viability of their application on the commercial production of sorghum in northern Tamaulipas.

### **Limitations**

The results of this study are valid under the test conditions reported in this study.

### **Conflict of interest**

The authors declare to have no conflict of interest.

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### **Contribution of authors**

K Kuri-Aguirre developed experiments, recorded the information, structured the data bases. J.G García-Olivares carried out the statistical analysis and interpretation. M Leal-Castillo and S Hernández-Delgado planned the study, wrote the manuscript and edited the manuscript.

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