Original paper Open Access

Inoculation of *Sphacelotheca reiliana* Spores to Identify Maize Germplasms Susceptible to Head Smut

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Keywords: *inoculation method, hybrid, maize disease, Zea mays*

Abstract

This study aimed to improve the inoculation procedure with *Sphacelotheca reiliana* to identify germplasms susceptible to head smut of maize. During Phase 1, 1.5 g of spores were introduced into a substrate roll, which was then sown at 15, 20, 25, and 30 days after inoculation in a spore-free field. During Phase 2, 15 hybrids were planted on three dates in a field with head smut disease record. The tassel and ear were examined, and the number of infected plants was registered. In Phase 1, all hybrids showed the highest infection rates 25 days after inoculation. In Phase 2, the highest infection percentage was observed on the first date (32.5%). Experimental hybrids 1 and 2 and hybrid DK357 showed the highest infection percentages, whereas hybrids DK2037 and DK2061 showed the lowest infection percentages. On the second date, 10 hybrids were susceptible to infection, while on the third date, the hybrids showed low infection percentages or were not infected. The disease response in the Cimarron, Experimental 3, and Antelope hybrids was relatively constant over the first two planting dates. The experimental hybrids and P4063W showed high infection percentages. On the first date, the Experimental 3, Experimental 2, P4063W and Experimental 1 hybrids showed infection percentages of 77, 65, 62 and 46%, respectively. On the second date, these percentages changed to 49, 35, 41 and 48%, respectively. The proposed inoculation method is more effective and can be employed a lower cost and with less infrastructure than previous methods.

Abbreviations

GLIMMIX: Generalized linear mixed model.
QTLs: Quantitative Trait Loci
SEM: Standard Error Mean
LSD: Least Significant Differences

Introduction

Head smut of maize is a disease of worldwide economic importance that affects corn. This disease is caused by the biotrophic pathogen *Sphacelotheca reiliana*, which infects maize by invading the root during the early seedling stage and destroys male and female inflorescence by replacing these structures with smut galls composed of powdery spore masses (Zhang *et al.*, 2013; Qiu *et al.*, 2021). Head smut has been reported in all corn-producing areas of the world, albeit with different levels of contamination, resulting in enormous economic losses to farmers (Yu *et al.*, 2014). In Mexico, in regions where maize is grown, head smut incidence has been estimated to range from 0.1 to 40% (Ramírez Dávila *et al.*, 2011). The states of Jalisco, Durango, Hidalgo, State of Mexico, among others, have detected the presence of this disease.

Chemical pathogen control of head smut is based on treating the seed with systemic fungicides; however, this is expensive and does not completely eliminate the fungus (Wright *et al.*, 2006; Aquino Martínez *et al.*, 2011; Álvarez Cervantes *et al.*, 2016). A more feasible and economical alternative form of disease management is creating genetic resistance, although it is necessary to develop a high-yielding, tolerant maize hybrid (Aquino Martínez *et al.*, 2011; Zuo *et al.*, 2015; Álvarez Cervantes *et al.*, 2016). Therefore, effective inoculation techniques are required to promote infection in maize by this pathogen.

Various artificial inoculation methods have been tested, which range from sowing seeds with spore-soil mixtures to using different spore inoculation procedures within seeds (Quezada Salinas A *et al.*, 2013; Quesada Salinas *et al.*, 2017; Marquez Licona *et al.*, 2018). To date, these techniques have yielded highly variable infection percentages, which limits their applicability and reproducibility in the field. In order to select maize varieties that are resistant to head smut disease, the results of artificial inoculation techniques must match those obtained under conditions of natural infections. To this purpose, it is necessary to develop an effective inoculation procedure. The aim of this study was to develop a procedure for the inoculation of head smut in maize with *Sphacelotheca reiliana* to identify maize germplasms that are susceptible to the disease.

Materials and methods

Experimental sites

Two study sites in Mexico were selected, which consisted of two phases. Phase 1, took place during the 2014 winter cycle in San Juan de Abajo, Nayarit, which had no history of head smut disease. Phase 2, took place during the 2015 summer cycle in Nextipac, Jalisco, in which endemic head smut disease has historically been present. Temperature and rainfall data for the experimental sites were obtained from the Weather Spark website (*https://weatherspark.com*).

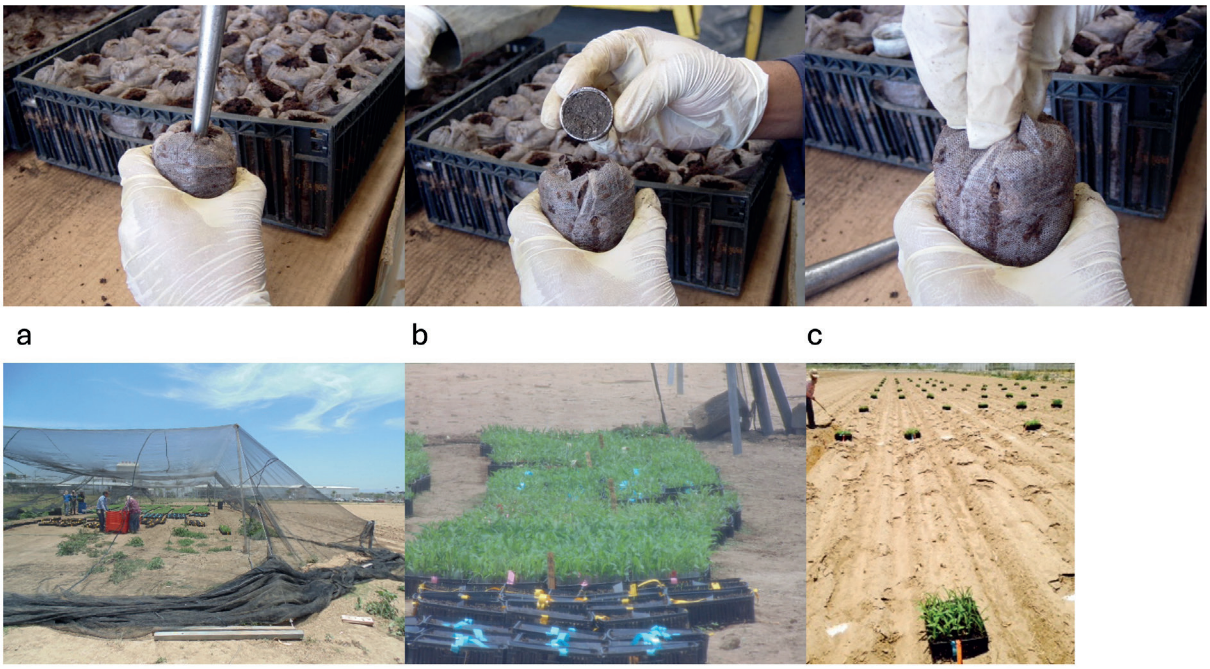
Complete randomized blocks with five replications (four inoculations and one control) were performed using a subdivided plot arrangement. In the main plot were the sowing dates and the genotypes in the sub-plot. The experimental plot consisted of 4.5m2. Two rows were made, with 25 plants per row. The separation between seeds was 8.3. Each row was 3 m long, with 0.75 m between rows.

Plant material

The tolerance or susceptibility to head smut was evaluated in three experimental hybrids and 12 commercial hybrids from ASGROW (Garañon, Cayman, Boa, Antelope, Cimarron, and A7573), DEKALB (DK2037, DK390, DK2061, and DK357), and Pioneer (P3164W and P4063W). Of these, 10 commercial hybrids are marketed in the Jalisco area, while two commercial hybrids are marketed in the tropical zone.

Inoculation and sowing method

For both phases, the number of spores per gram was determined following the methodology of French and Hebert (1980). Spore inoculation was conducted with rolls of a pre-moistened substrate (5 cm in diameter x 10 cm long), which consisted of 50% coconut fiber and 50% vermiculite. The moisture retention and porosity of the substrate ranged between 40 – 65% and 75 – 85%, respectively. A total of 1.5 g of spores were introduced into the substrate roll that was subsequently left in the field under a shade mesh with anti-bird protection and black plastic to maintain average humidity and temperature conditions of 50% and 27°C, respectively. Seed



a b c d e f

Fig. 1 - Inoculation method. The first step after mixing the substrate is to hydrate it, subsequently, a 5-cm deep hole is dug (a), and the spores are introduced (b). Then, the substrate roll is closed (c). After which, it is installed in the field under shade mesh (d and e). Planting in the field (f).

sowing was performed on the scheduled days, depending on the phase of the experiment. Subsequently, a direct transplant in the field was conducted at the V2 stage of plant development (Figure 1).

The study was divided into two phases; the first phase was to determine the optimal day of inoculation to ensure the germination of the spores and their highest percentage of infection. Phase 2, determine the effect of planting dates and the probability of infection of the hybrids.

Phase 1

Three hybrids (Garañon, DK357, and P4063W) and a pure line were planted during Phase 1 in soil free of *Sphacelotheca reiliana* spores. The planting dates of this phase were 15, 20, 25, and 30 days after substrate inoculation. Drip irrigation was used in this site to control the water provided to the plants and to avoid possible spore spread.

Data were collected after harvesting the cobs. Plants that showed evidence of disease were eliminated before they matured to avoid spore proliferation in the field. The substrate roll, soil, and roots were extracted from each plant, both with and without the disease.

Phase 2

For Phase 2, seeds were planted in a field where the endemic presence of head smut of maize has been reported. Seed sowing was conducted with the results of Phase 1 with regard to the number of days of substrate inoculation.

Seeds were sown on three dates, with an interval of 15 calendar days between dates. A total of 15 hybrids with five repetitions (four with inoculation and one without inoculum) were planted. Auxiliary irrigation was provided during the first two sowing dates to establish seedlings. At this site, infected ears were harvested for data collection and spores were collected for storage.

Evaluations

Head smut was evaluated and scored for each plant from the milk stage until physiological maturity. The tassel and ears were examined to characterize malformations. In cases when malformations were not easily visualized, the ears were opened to detect the characteristic deformation of the ear. The total number of plants infected per hybrid was determined. To avoid contamination in the experimental field during Phase 1, all ears were split before maturity to identify and assess infections.

The signs considered as evidence of the disease were deformation in the tassel or black powdery mass or both. The ear could be small and soft, and a mass of spores may partially or totally replaced the kernel

Data Analysis

The results obtained in Phase 1 were analyzed by percentages of infection of the materials on the different planting dates, and standard error of the mean (SEM). In phase 2, infection percentages of diseased plants of each hybrid were obtained at the different planting dates. Generalized linear mixed model (GLIMMIX)

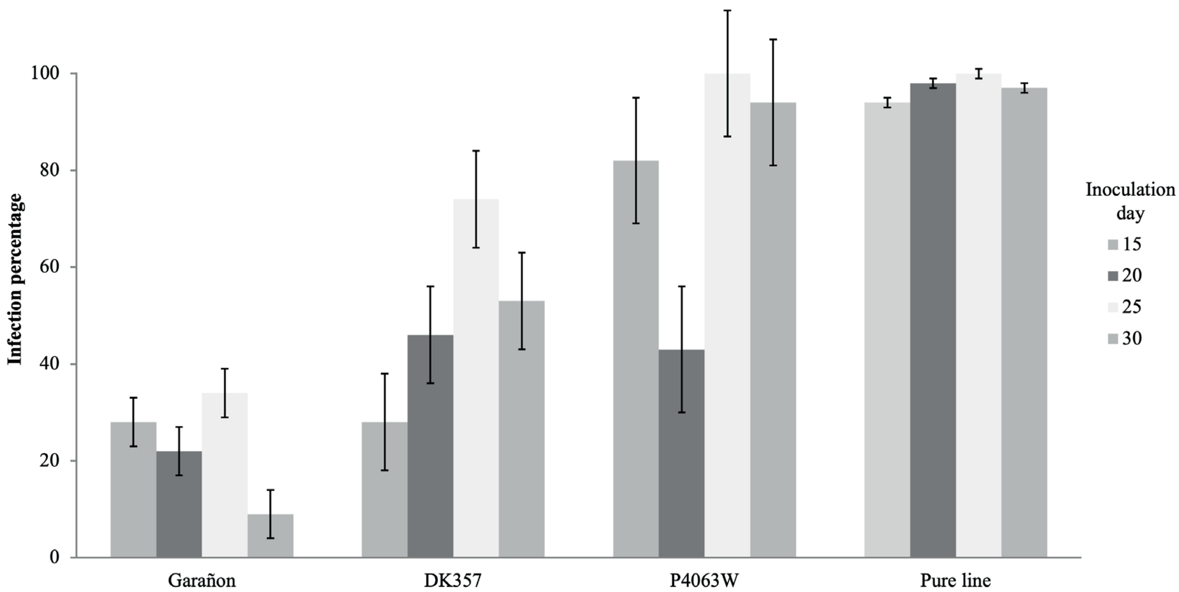


Fig. 2 - Infection percentages during Phase 1. Infection percentages of the three hybrids identified as susceptible and those of the pure line. Seeds were planted on four sowing dates (15, 20, 25, and 30 days after inoculation in the substrate). Bars represents the standard error of the mean

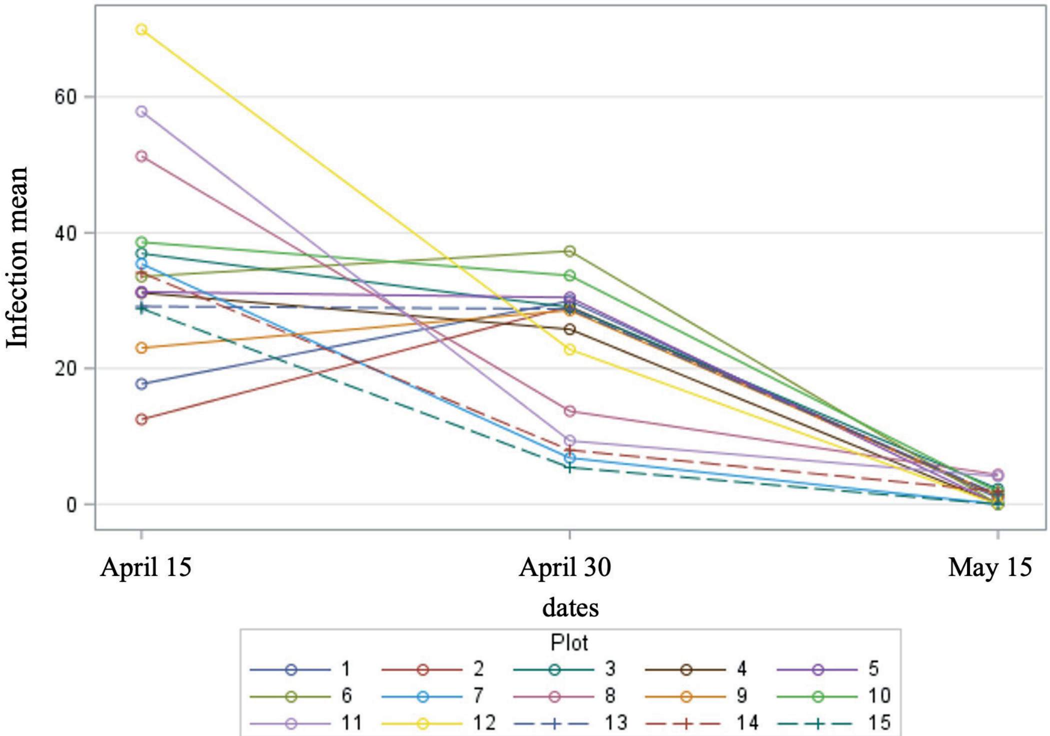


Fig. 3 A - Mean infection percentage by date. The infection rate is very high on the first date and decreases over time. 1, A7573; 2, Garañon; 3, Cayman; 4, Cimarron; 5, Antelope; 6, Boa; 7, DK2061; 8, DK357; 9, DK2037; 10, DK390; 11, Experimental 1; 12, Experimental 2; 13, Experimental 3; 14, P4063W; 15, P3164W.

analysis and two-way ANOVA was performed for the presence of head smut at planting dates and the interaction between hybrids and sowing dates. The level of significance was set at $p<0.001$.

Results and Discussion

Spore viability

The germinated spore count, an average of 18% of the spores per gram were germinated, which was low. However, when the relative humidity increased, so did the number of germinated spores on average 40%. Maximum humidity of 65% is recommended for substrate preparation, and for this reason, the low germination value was accepted.

Substrate inoculation Phase 1

The highest and lowest temperatures in the San Juan de Abajo municipality of 32°C and 15°C were recorded in November and December 2014, respectively. Rain was also recorded at the beginning of November. Both these factors helped influence the success of infection during planting.

Evidence of disease due to *Sphacelotheca reiliana* was observed in both the susceptible inbred line and experimental hybrids. The pure line showed a higher incidence of infection, followed by the hybrids P4063, DK357, and Garañon. Moreover, evidence of the disease was observed on the four planting dates. The pure line showed close to 100% infection for all four planting dates (15, 20, 25, and 30 days after inoculation). The number of infected plants of the P4063W hybrid decreased when planted 20 days after inoculation, whereas the number of infected plants of the DK357 hybrid increased when planted 25 days after inoculation (Figure 2). These two hybrids have already been

Table 1 - GLIMMIX analysis for planting dates in 2015 in Nextipac, Zapopan, Jalisco.

Date	Estimated value	Standard error	DF	t Value	Pr > |t|	Half	Mean Standard Error
April 15	3.4843	0.1142	6	30.52	< 0.0001	32.5998	3.7221
April 30	2.9542	0.1165	6	25.36	< 0.0001	19.1859	2.2345
May 15	-8.4995	114.47	6	-0.07	0.9432	0.000204	0.02330

(Null Hypothesis: Head smut percentage for dates = 0)

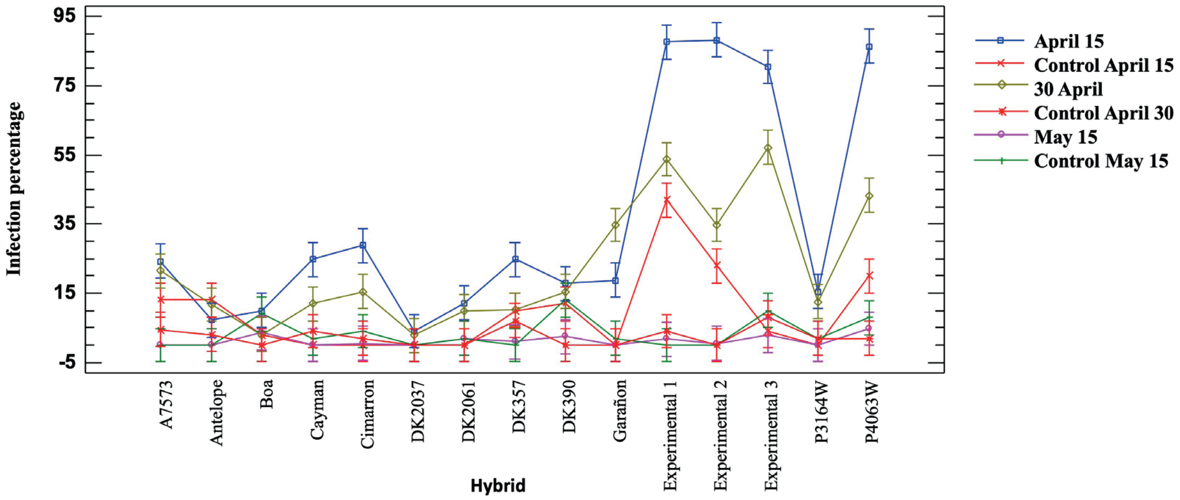


Fig. 3 B - Mean infection percentage by date. Behavior between hybrids by date separated with and without inoculum.

proven to be susceptible to head smut.

All hybrids showed a higher incidence of *Sphacelotheca reiliana* disease for the third planting date (25 days after inoculating the substrate; Figure 2). This result agrees with what was reported by Quezada Salinas *et al.* (2013), who observed molecular evidence of the mycelium in plant tissues of seedlings developed from seeds inoculated with *Sphacelotheca reiliana* and collected after 25 days of sowing, in addition to evidence of the mycelium in the host tissue and the presence of sori at the end of the experiment. Therefore, this result was used to plan Phase 2.

Phase 2

Environmental conditions influence the development of head smut disease, with dry and cold climates at the beginning of the season favoring the disease. During these conditions, advancement through the initial stages of the disease is promoted and seedling growth is retarded. Once a plant becomes infected, dry and hot climates favor the development of *Sphacelotheca reiliana*. In particular, soil temperatures between 21–30°C and humidity values between 15–25% favor the development of the infection (Martínez C *et al.*, 2000; Ramírez Dávila *et al.*, 2011; CIMMYT, 2014).

The temperature records for Nextipac, Jalisco, indicated that temperatures in April and May 2015, which ranged between 10–34°C, were the highest recorded for the year. During the first and second sowing dates (April 15 and 30), the highest recorded temperature was 31°C, whereas temperatures reached 34°C during the third planting date (May 15). Humidity values ranged between 12–60%, which were also the lowest recorded for the year. Although the range of recorded temperatures was ample, the temperatures favored disease progression, despite being different from those of Phase 1. Germination of *Sphacelotheca reiliana* was conducted under controlled conditions; the disease progression was only allowed in a field in which the disease had already been recorded.

It is well known that many biological variables are not normally distributed (Pedrosa *et al.*, 2015), and thus the normality of the data was evaluated with goodness-of-fit tests. As expected, the results of the goodness-of-fit tests indicated that the data were not normally distributed, even after logarithmically transforming the data. Thus, we concluded that the observed data were non-normally distributed in nature. Therefore, the GLIMMIX procedure was used with the original percentage data considering a negative binomial distribution, which was adjusted appropriately with a chi-square value of 0.77 (values < 1.0 are recommended) (Stroup, 2012).

These results agree with the field study by Sanchez Pale

Table 2 - Significance of F-values in the analysis of variance with GLIMMIX.

Effect	Num FD	give DF	F Value	Pr > F
Hybrid	14	126	5.67	**< 0.0001**
Date	2	6	5.29	0.0474
Hib.* Date	--	126	61.56	**< 0.0001**

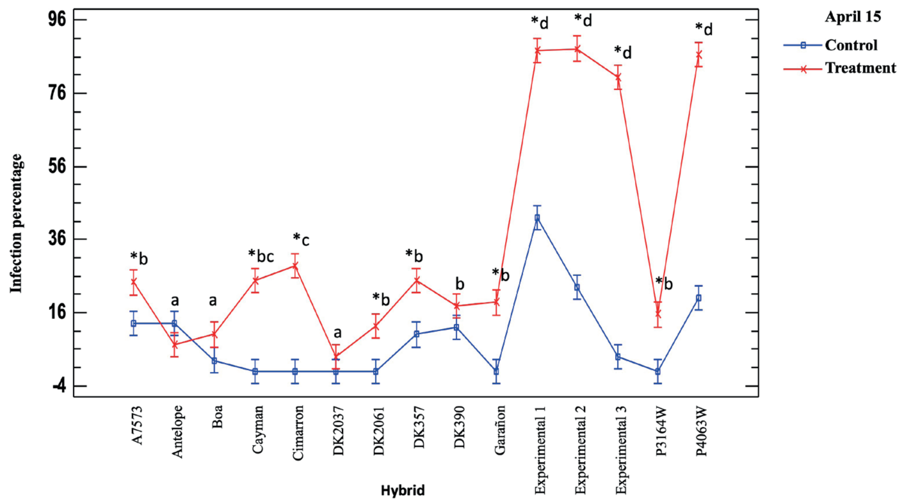


Fig. 3 C - Mean infection percentage by date. Statistical comparison among hybrid of the date of April 15.

Different letters indicate significant differences among hybrids according to the least significant differences (LSD) mean comparison test (p ≤ 0.001). The asterisks denote a significant difference between treatment and control for each hybrid.

et al. (2011), who showed that *Sphacelotheca reiliana* infection did not show a uniform probability distribution. These authors did not find a relationship between the infection rates in the field with what was estimated, suggesting that environmental conditions or susceptible maize genotypes may have favored disease expression. In addition, harmful organisms often show variable densities. Similar results with normal distributions have been reported in other crops (Roumagnac *et al.*, 2004; Mouen Bedimo, 2007).

With the information obtained from the experiments conducted in Nextipac, Jalisco, it was determined that the highest infection percentage (32.5%) occurred on the first planting date (April 15), followed by averages of 19.1% and 0.0002% on the second (April 30) and third (May 15) planting dates, respectively. When analyzing the infection percentages of the 15 hybrids, significant differences were observed among planting dates (Table 1). Moreover, differences between hybrids were significant, as well as the interaction between hybrids and dates (Table 2).

When analyzing the head smut percentages in maize, significant differences were observed between hybrids (< 0.0001) as well as in the interaction between hybrids and sowing dates (< 0.0001). In addition, significant differences were present on the first (April 15; < 0.0001)

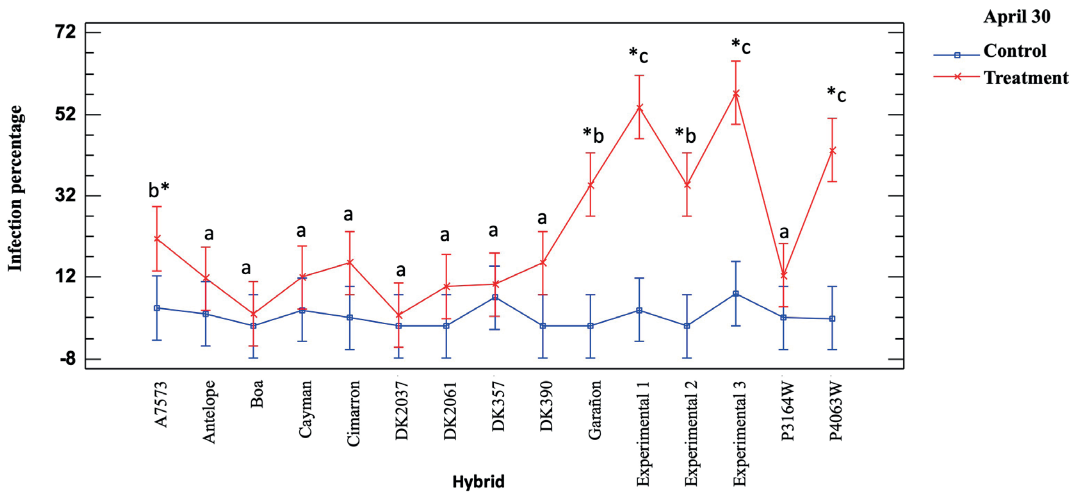


Fig. 3 D - Mean infection percentage by date. Statistical comparison among hybrid of the date of April 30.

Different letters indicate significant differences among hybrids according to the least significant differences (LSD) mean comparison test (p ≤ 0.001). The asterisks denote a significant difference between treatment and control for each hybrid.

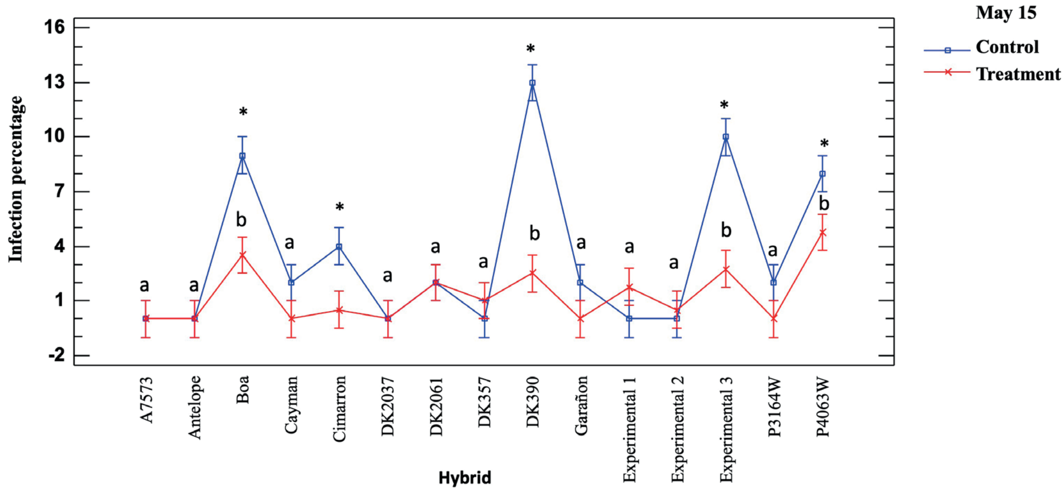


Fig. 3 E - Mean infection percentage by date. Statistical comparison among hybrid of the date of May 15.

Different letter indicate significant differences among hybrids according to the least significant differences (LSD) mean comparison test (p ≤ 0.001). The asterisks denote a significant difference between treatment and control for each hybrid

and second (April 30; < 0.0001) sowing date, although not on the third sowing date (May 15; 0.9432; Table 1)

As shown in Figure 3, a high infection percentage was present on the first date in most hybrids. On the second date, the infection percentage was lower and more concentrated and decreased considerably on the last date (Figure 3a). This may be related to the temperature and humidity conditions during the experiment. On the first date, the temperature and humidity conditions were more in line with those necessary for disease progression. As the experiment progressed, both temperature and humidity increased, which may have reduced the signs of *Sphacelotheca reiliana* disease.

On the first date, when high infection percentages were observed in the hybrids, wide-ranging responses to *Sphacelotheca reiliana* disease were detected among the hybrids (p ≤ 0.001). Based on these differences, the hybrids were classified into three groups: a) highly susceptible (Experimental 1, Experimental 2, and DK357), b) susceptible (Cayman, Boa, Antelope, Cimarron, DK390, DK2061, P3164W, P4063W, Experimental 3) and c) uninfected (Garañon, A7573, and DK2037; Figure 3). On the second date, two hybrid groups were present: a) susceptible (Garañon, Cayman, Boa, Antelope, Cimarron, A7573, DK2037, DK390, Experimental 2, Experimental 3) and b) non-infected (P3164W, DK2061, P4063W, Experimental 1, and DK357). On the third date, all hybrids were classified as uninfected with very low percentages of infection (Figure 3). Lastly, the disease responses of the Cimarron, Experimental 3, and Antelope hybrids were more or less constant over the first two planting dates (Figure 3).

When the infection percentage of the hybrids was evaluated together with the controls (the control was subtracted from the hybrid under evaluation; Figure 4, p ≤ 0.001), it was observed that hybrid DK2037 showed the lowest infection level, which was the same on both the first and second date (4 and 3%, respectively). Hybrid DK2061 showed a similar behavior on both dates (12 and 10%, respectively). However, the Boa hybrid showed contrasting behavior. On the first sowing date, an infection rate of 7% was observed, whereas this decreased to 3% on the second sowing date. On the last sowing date, the control showed a 9% infection rate, while sowing with the inoculum showed a 1% infection rate.

Overall, the experimental hybrids and hybrid P4063W showed the highest infection percentages. Moreover, on April 15, the highest infection rates were recorded, with Experimental 1, Experimental 2, Experimental 3, and P4063W showing infection percentages of 46, 65, 77 and 62%, respectively. On April 30, these percentages changed to 48, 35, 49 and 41%, respectively (Figure 4).

Marquez Licona *et al.* (2018) reported an infection percentage of 54% in one line and 70% in hybrid AZ41801 in the field with their inoculation method, which is more labor-intensive than what we report here. In our study, the hybrid P4063W obtained an infection percentage of 62%, and the experimental hybrids achieved infection percentages as high as 77%.

The identification of genotypes that possess resistance genes can be achieved by using visual assessments and categorizing them into different groups based on resistance levels. Although visual assessments are simple and easy to perform, they lack precision and specific-

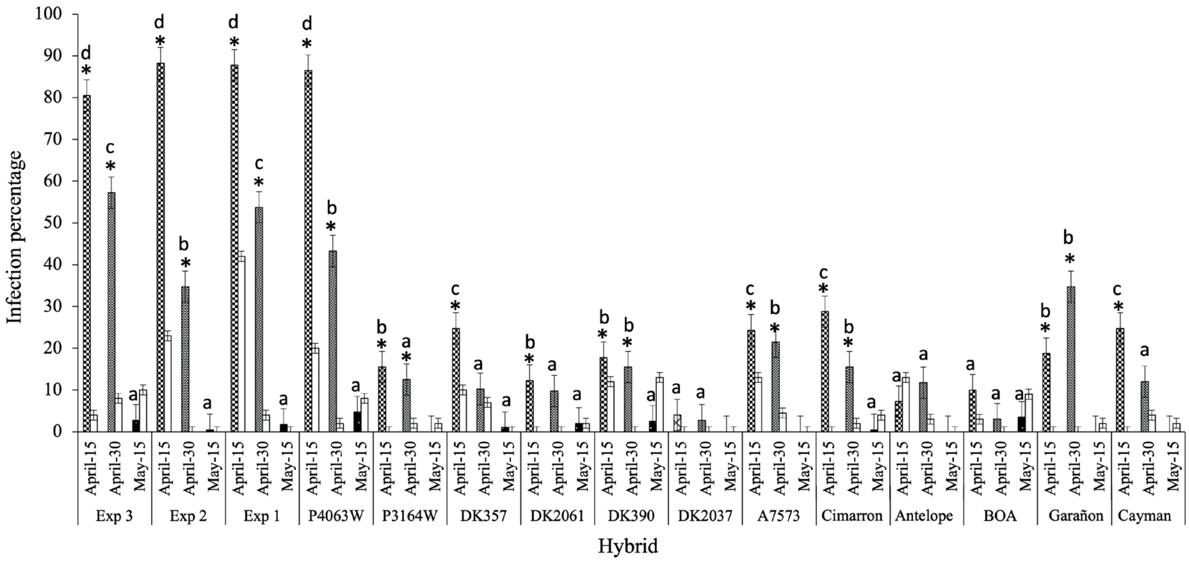


Fig. 4 - Infection percentages the hybrids and controls. Columns denoted by a different letter indicate significant differences among infection times according to the least significant differences (LSD) mean comparison test (p ≤ 0.001). Columns denoted by asterisks denote a significant difference between treatment and control for each hybrid (white columns). Bars represent standard error of the mean.

ity (Narayanasamy, 2008; Lübberstedt, 2013). Working with hybrids, such as those mentioned above, to understand the mechanisms of gene expression and comparing their gene expression patterns with those of disease-resistant hybrids can provide a clearer path forward to obtaining a maize variety with high disease resistance and agronomically favorable traits without sacrificing corn yield.

Head smut resistance is considered a quantitative trait controlled by additive, dominant, and epistatic effects (Qiu *et al.*, 2021). Several quantitative trait loci (QTLs) have been identified that are associated with head smut resistance. In particular, the *ZmWAK gene* has been associated with disease resistance and reduces disease incidence by approximately 25% (Zhang *et al.*, 2021; Zhu *et al.*, 2021). Most QTLs only make small genetic contributions that help to reduce disease severity (Zhu *et al.*, 2021). Understanding these genes and their roles in disease resistance can be useful for molecular marker-assisted selection in maize breeding programs (Qiu *et al.*, 2021; Zhang *et al.*, 2021).

In this study, different response levels among hybrids were observed, allowing for the ones that responded favorably to *Sphacelotheca reiliana* inoculation and disease progression to be visually identified. The results of previous studies with variable infection percentages are not comparable with those of this study. In this study, 15 different genotypes were included with wide-ranging variability, whereas previous studies have only included a few genotypes. Quezada Salinas *et al.* (2013) used seed inoculation and cultivated seeds under greenhouse conditions, whereas Baggett and Koepsell (1983) used different maize hybrids as well as seed inoculation. Besides, more infrastructure is required than the used in this study.

In this study, substrate inoculation and controlled humidity and temperature conditions produced the optimal conditions for pathogen germination. The resulted in the infection of maize hybrids, although with variable infection percentages. After which, the disease developed due to stressful conditions. Martínez *et al.* (2003) reported that the available agronomic data on root infection by Sphacelotheca reiliana indicates that temperatures of 21–30°C are optimal for infection in maize in the field. In addition, the water potential is an abiotic factor that affects microorganism development in soil. Studies of disease severity have shown that more maize seedlings are infected when growing in soil with low water potential (Matyac and Kommendahl, 1985; Martínez *et al.*, 2003).

The Cimarron and Antelope commercial hybrids and Experimental hybrid 3 showed the best responses to the disease. Although these hybrids cannot be classified as tolerant, the balance between infection and corn yield should be evaluated. We recommend using the Cimarron and Antelope commercial hybrids in the first half of April. Although there is a greater probability that the disease will manifest itself during this time, the corn yield should be higher. If drought stress can be avoided, the incidence of *Sphacelotheca reiliana* disease decreases considerably. The disease is less likely to appear when planting in May, although the corn yield should decrease considerably during this time.

Resistance is recognized as the most desirable and cost-effective method to manage the disease. Identifying cultivars with acceptable levels of resistance to major diseases is considered the best approach, as these can reduce or eliminate the associated expenses and effects of chemical, physical, biological, cultural, and regulatory control methods (Lübberstedt *et al.*, 1999; Narayanasamy, 2008).

Conclusions

In this study, it was possible to further approaches to managing head smut in the field by identifying hybrids that are highly susceptible to the disease as well as those with better infection responses. We found that the optimal date for spore germination with the highest percentage of infection is 25 days after inoculation prior to sowing. Experimental hybrids 1 and 2 and the commercial DK357 hybrid were identified as being susceptible to head smut, as they were unable to evade infection by *Sphacelotheca reiliana* on the date with the best corn yield. The inoculation method proposed in this study allowed us to detect the best planting dates with the inoculum as well as the susceptible hybrids. A greater quantity of materials can be analyzed at low costs without the need to invest in complicated infrastructure or utilizing labor-intensive techniques

Acknowledgments

We thank José de Jesús Sánchez Gonzáles and Rosalba Mireya Hernández Herrera for his contribution to the work with the statistical analyses.

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