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Artificial inoculation of maize seeds with *Sporisorium reilianum* f. sp. *zeae*

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Abstract

The objective of this research was to identify an inoculation method of *Sporisorium reilianum* f. sp. *zeae*, causal agent of the head smut of maize, which allows a high and consistent percentage of infection, for reliable selection of germplasm with genetic resistance to this pathogen. Seeds of the hybrid AZ 41801 were inoculated with teliospores impregnated using as adherents: 1% (w/v) sodium carboxymethylcellulose (NaCMC) 1%, ADH®, 2% (w/v) sodium alginate 2%, 50% (w/v) brown sugar 50% (w/v), 2% (w/v) agar – agar, 10% (w/v) grenetin and a treatment 1: 200 (v/v) of inoculum: soil 1: 200 (v/v). Treated seeds were planted in a greenhouse in 40 x 40 cm polyethylene pots with sterile soil. In 2015, grenetin as adherent, induced 96.3% of diseased plants in greenhouse. Morphological and molecular characterization of the pathogen was carried out before the inoculation and after the final evaluation. This method of inoculation was evaluated in 2016 in field conditions in Mixquiahuala, Hgo. A total of 160 lines C2-S3 from a bmaize population improvement program were inoculated with teliospores of *S. reilianum* f. sp. *zeae* using 10% grenetin as adherent. The incidence of the disease in both experiments planted in 2015 and 2016 was evaluated 45 days after flowering, with direct observation of symptoms and signs in male and female inflorescences. One line showed 54.5% of infection. The hybrid AZ 41801 reached 70.7% of diseased plants. It is suggested to use 10% grenetin as adherent in the inoculation of maize seeds with teliospores of *S. reilianum* f. sp. *zeae* for the selection of disease resistant germplasm.

KeyWords: *Zea mays, head smut, artificial inoculation with teliospores, grenetin, genetic resistance.*

Introduction

The head smut of maize, caused by *Sporisorium reilianum* f. sp. *zeae*, is an important disease under certain conditions that favor its development, reaching incidences of up to 80% (Frederiksen, 1977). Several conditions benefit the development of the pathogen, such as genetic vulnerability of cultivated hybrids (Fehr, 1993), soil type, fertility level, planting depth, soil temperature and soil moisture (Matyac and Kommedahl, 1985a). The head smut of maize is a systemic and soil-borne disease (Lübberstedt et al, 1999). The fungus infects maize during the emergence of seedlings through teliospores present in the soil (Xu et al, 1999). Germinated teliospores develop a basidium of 4 cells (promicelium), where several haploid basiodiospores are produced. Later, through pairing of compatible cells the parasitic phase of the fungus is formed (Martinez et al, 1998) and the infective hypha penetrate the roots of maize seedlings (Martinez et al, 2001). The mycelium grows systemically within the meristem zone until, after sporogenesis, the inflorescences are replaced by black sori filled with teliosporas (Xu et al, 1999)

The use of chemical products in the seed is the most used control measure for the management of the head smut of maize (Fullerton et al, 1974). Seed treatment with propiconazole can reduce up to 70% the percentage of diseased plants, compared to untreated seed (Wright et al, 2006). However, the use of disease resistant germplasm can be used in an integrated disease management program. Even when chemical control is feasible (Wang et al, 2008), the development of disease resistant germplasm materials is the best strategy to control the disease. To develop resistant germplasm, it is important to have an efficient inoculation method which considers the nature of the crop, the stage of development at the time of the evaluation, as well as the life cycle and genetic variability of the pathogen. This ensures the evaluation of a large amount of germplasm and having a high percentage of infection which allows a discrimination between resistant and susceptible materials (Niks et al, 2011).

The methods of inoculation so far developed (Metha, 1967; Baggett and Koepsell, 1983; Stromberg et al, 1984; Matyac and Kommedahl, 1985b; Whythe and Gevers, 1987; Craig and Frederiksen, 1992; Pradhanang and Ghimire, 1996; Quezada-Salinas et al, 2013), have

been the basis for several investigations, but the efficiency of these methods in germplasm selection under field conditions is not clear as the percentages of induced infections vary considerably, limiting their use in a breeding program of disease resistance.

The germination rates of *S. reilianum* teliospores vary from 15% to 35% (Potter, 1914; Osorio y Frederiksen, 1998; Quezada-Salinas et al, 2013), with 60 to 90% of germination under specific conditions of storage and incubation (Pai and Pan, 1964). Limiting the efficiency in reproducing the percentages of infected plants, making impossible an adequate evaluation of germplasm with resistance to the pathogen.

At present, there is a need for head smut resistant germplasm for areas where the pathogen has become important. Therefore, the objective of this research was to develop an efficient inoculation technique of maize seeds with teliosporas of *S. reilianum* f. sp. *zeae*, for the selection of disease resistant germplasm.

Materials and methods

Inoculum

To select a source of inoculum with a high percentage of germination, during the year 2014 collections of tassels and ears infected with *S. reilianum* f. sp. *zeae* were done, obtaining 2 collections from the state of Guanajuato, 10 from Hidalgo, 6 from Mexico and 1 from Zacatecas. The teliospores were removed from the sori and passed through a 117µ mesh (Mont Inox®), stored in plastic containers with $CaCl_2$ (J.T. Baker and Macron Fine Chemicals® 94%), and stored at 20 ± 2 °C until use. The viability of the inoculum was verified prior to inoculation of the seed as described by Quezada-Salinas et al, (2013), selecting as inoculum the collection with the highest germination percentage.

The morphological characterization of the inoculum was done by observing permanent mounts of the pathogen. For this, 100 spores were characterized using an optical microscope. Measurements were done under a light microscope at 100 X (CX31RBSFA, Olympus®). The molecular characterization was done from monobasidial cultures, amplifying the ITS region (Internal Transcribed Spacer) and part of the LSU rDNA (Large Subunit Ribosomal DNA) region using the ITS1F / NL4 primers.

To produce monobasidial cultures, teliospores of *S. reilianum f.* sp. *zeae* previously disinfected in an aqueous solution 1.5% of $CuSO_4$ (w/v) for 24 h (Holliday, 1974), washed with three changes of sterile distilled water and recovered by vacuum using a Büchner funnel with sterile filter paper grade 1 (Whatman ®). A 50.000 teliospores mL^{-1} suspension in sterile distilled water was prepared and deposited 1 mL per Petri dish with Potato-Dextrose-Agar (PDA) medium, distributing the suspension on the surface of the medium and removing the excess water on sterile absorbent paper. The dishes were incubated in dark at 25 °C for 24 h. From the germinated teliospores, individual basidia were transferred to dishes with PDA. Monobasidial cultures grew for 15 days and one monobasidial cultures was selected for DNA extraction.

Total DNA extraction from the monobasidial culture was performed with Ultra Clean® Microbial DNA Isolation Kit MoBio Laboratories Inc. DNA integrity was checked on a 1% agarose gel and the amount of DNA was determined by spectrophotometry at 260 nm absorbance with a (Nanodrop® ND-1 V 3.2.1). A reaction mixture of 25 µL was used, My Taq buffer 1X, 10 pm of each primer, 1U of MyTaq™ DNA Polymerase (Bioline Germany) and 100 ng DNA. The amplification was performed in an Eppendorf ® thermocycler, Mod. Mastercycler Pro with an initial denaturation of 94°C for 4 min, followed by 35 cycles at 94°C for 1 min for denaturation, 55°C 1 min for alignment and 72 °C for 1 min for extension, plus a 72°C cycle for 10 min for final extension.

The amplified products were visualized through 1% agarose gel electrophoresis at 120 volts for 30 min. To estimate the size of the amplified fragment, the molecular marker Pst I (Microzone ®) was used, which indicates 28 different sizes of fragments ranging from 15 base pairs to 11.49 Kilobases. After electrophoresis, the gel was analyzed with a photodocumentator Chemi Genius 2 Bio Imaging System (Syngene®). PCR products were purified as described by Kirby (1965) and sent to sequence to Macrogen Europe, Amsterdam, The Netherlands.

The quality of the sequences was determined by the electropherograms using the 4peaks® Nucleobytes software (Griekspoor and Groothuis, 1994), while the sequences were aligned using the UGENE software (Okonechnikov et al, 2012). The obtained sequences were compared in the GENBANK database using the BLAST tool (Altschul et al, 1990) from the National Center for Biotechnological Information (NCBI).

Evaluation of treatments in greenhouse

Maize seeds of the white endosperm commercial hybrids AS1503 (Aspros), AZ 41801 (Azteca) and Euros (Unisem) were used. The commercial fungicide treatment of the seeds was removed by washing them with a 1% solution of Rome detergent (Fábrica de Jabón La Corona S.A. de C.V.) by vigorously shaking. To inactivate the remaining fungicide present on the

seeds, they were treated with a solution of lime (pH 12) for 5 min. Subsequently, the seed was disinfested with a 1.5% solution of sodium hypochlorite (NaOCl) for 2 min, washed with three changes of sterile distilled water and dried at 22°C on sterile absorbent paper. Before inoculation of seeds, the effect of sodium carboxymethylcellulose (NaCMC), ADH, alginic acid, brown sugar, agar and grenetin on the germination percentage of the teliospores was determined; For this, 1 mL of the teliospore suspensions was added in Petri dishes with PDA with three replicates. The dishes were incubated in darkness at 25°C for 24 h and the percentage of germination was evaluated in three fields at 40X per dish. No detrimental effect on teliospore germination was found in any of the adherents, so they were used in seed inoculation treatments.

The treatments evaluated for the identification of an efficient inoculation technique of *S. reilianum* f. sp. *zeae* in maize seeds were: 1: control; 2: seeds were incorporated into the solution of 1% (w/v) NaCMC with $1.7x10^7$ teliosporas mL^{-1} for 1 min, recovered and dried at 23 2 ° C for 24 h. (Quezada-Salinas et al, 2013); 3; seeds were incorporated into the adherent ADH® (Cosmocel) with $1.7x10^7$ teliosporas per mL for 1 min, recovered and dried at 23 ± 2 ° C for 24 h; 4; seeds were incorporated one by one into 2% (w/v) sodium alginate with 1.7x107 teliosporas mL^{-1} for 1 min, recovered and placed in a 1.6% (w/v) solution of calcium nitrate ($Ca(NO_3)_2$). They were recovered in a Petri dish with sterile distilled water until the time of planting (Bashan, 1998); 5; the adherent used was brown sugar 50% (w/v); 6; 2% (w/v) agar-agar (w/v); 7; 10% (w/v) grenetin (w/v); in treatments 5, 6 and 7, seeds were incorporated into adherents for 1 min, recovered and in a Petri dish where they were completely covered with teliospores. The seeds were then placed in a Petri dish and dried at room temperature (23 °C) for 24 h; 8: seeds washed without fungicide protection were sown in pots with an inoculum-soil mixture 1:200 ($1.8x10^6$ teliospores/g^{-1} soil) (Matyac and Kommedahl, 1985b).

In 2015, inoculated maize seeds were planted in a greenhouse of the Colegio de Postgraduados, Campus Montecillo, Texcoco, Méx. (19°27'43.1"N,98°54'13.4" W) in 40x40 cm polyethylene pots with sterile soil used by Quezada-Salinas et al, (2013). After sowing, the pots were irrigated at field capacity. To increase the percentage of infection, constant irrigation was avoided during the early stages of seedling development (Wu et al, 1981; Mack et al, 1984). The plants were fertilized every 20 days using a nutrient solution of 5.000 ppm nitrogen (46-00-00). The humidity and temperature of the greenhouse was recorded with the help of an RHT10 EXTECH Instruments datalogger.

The incidence of the disease was recorded 45 days after flowering, counting the total number of plants per pot, and the number of infected plants in the different treatments. The tassels were evaluated by direct observation of signs and symptoms, while the ears were longitudinally sectioned to observe the internal presence of sori. The teliospores from infected plants were again characterized morphologically and molecularly to corroborate the identity of the pathogen.

The data obtained were analyzed based on the model of a spli-plot design, where the main plots were the hybrids, and the sub plots were the treatments, with three replications per treatment. Data was subjected to analysis of variance and multiple comparison of means by Tukey's honest significant difference method at a significance level of 5% using statistical software SAS Systems for Windows V 9.4. (SAS Institute Inc. 1999).

Validation of the best method in field conditions

After evaluation in the greenhouse, the best method of inoculation was validated in field conditions. It was decided to validate the method determining differences in resistance in homozygous lines. For this, 22 seeds of each of 160 white endosperm C2-S3 lines derived from a breeding program for genetic resistance to *S. reilianum* f. sp. *zeae* in the states of Mexico and Hidalgo were inoculated. Inoculated and noninoculated AZ 41801 hybrid seeds were used as check. Prior to inoculation, the viability of the inoculum used was checked.

During 2016, infection of the C2-S3 lines inoculated with *S. reilianum* f. sp. *zeae* was measured by planting them in the Ejido Cinta Larga, Mixquiahuala, Hgo (20°11'24.6" N 99°14'35.2" W). Inoculated seeds of each line were planted in a 3 m long rows, 0.80 between rows, at a depth of 5 cm and 14 cm between seeds. After sowing, the soil was irrigated by flooding avoiding constant irrigation during the early stages of seedling development to increase the percentage of infection (Wu et al, 1981; Mack et al, 1984).

The incidence of disease in each of the C2-S3 lines was recorded 45 days after flowering. Diseased plants were those that presented symptoms in the tassel, ear or both. The response to infection of *S. reilianum* f. sp. *zeae* of the 160 lines was separated by the percentages of diseased plants, considering the ranges 0%, 0.1 - 10%, 11 - 20%, 21 - 30%, 31 - 40%, 41 - 50%, 51 - 60% and 60% infection.

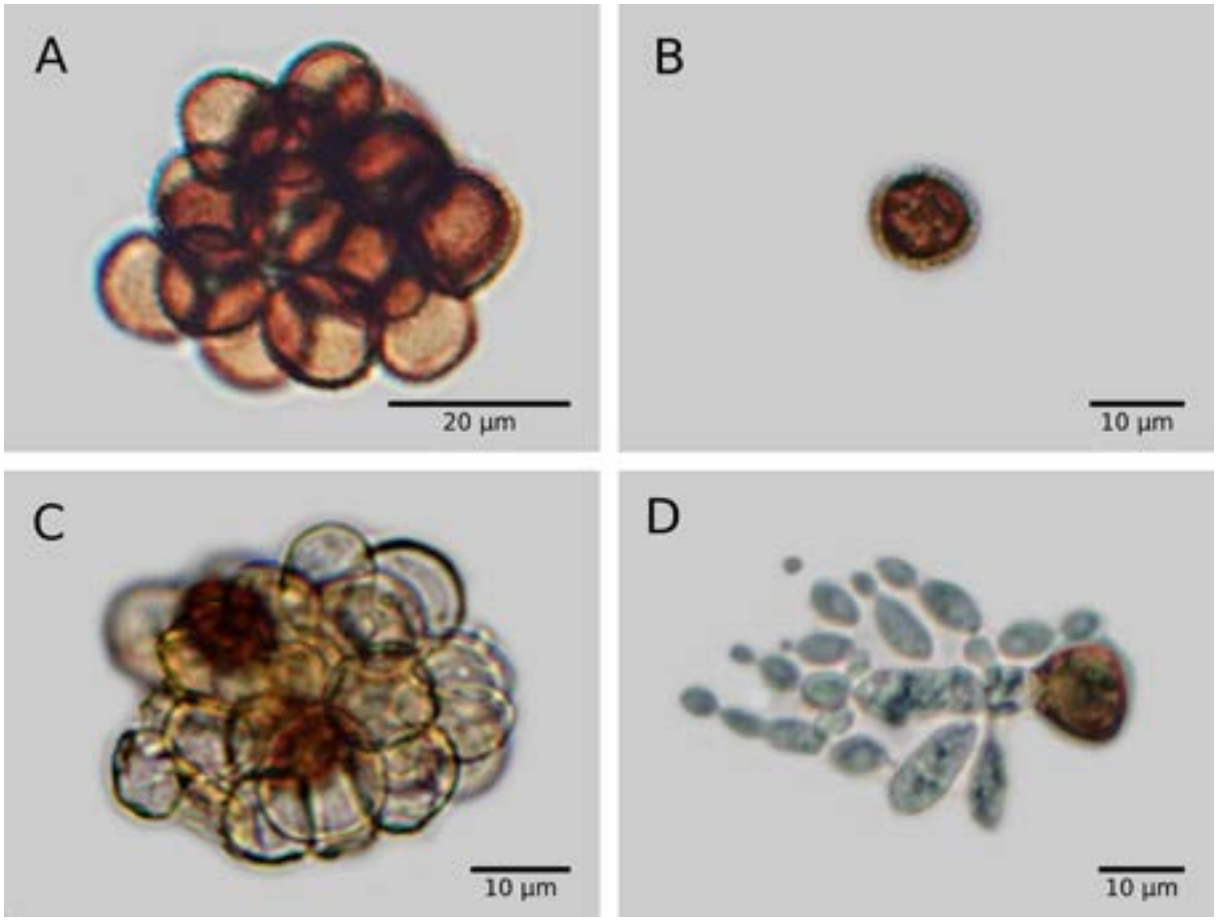

Figure 1. Structures of *S. reilianum f. sp. zeae*. A. mass of teliospores, B. Teliospore, C. Grouped sterile cells. D. Germinated teliospore.

Results and Discussion

Inoculum

After 24 h, in Petri dishes with PDA at 25 °C, 18 of the 19 samples tested showed teliospore germination rates between 5 and 19%. In contrast, the teliospores collected in a lot of maize of the Cardenal hybrid (Asgrow®) in the municipality of Tenango del Valle, Mex., reached 45.3% germination, which was chosen as source of inoculum for this research.

Spore disinfestation with 1.5% $CuSO_4$ inhibited the development of contaminating fungi present in sori, such as *Fusarium* spp. The percentage of teliospore germination in the sample used as inoculum after 24h of incubation was higher than those reported by Potter (1914), Osorio and Frederiksen, (1998), and Quezada-Salinas et al, (2013) who obtained maximum germinations of 15, 28 and 35.4%, respectively. This variation in percentages of germination may affect the percentages of infected plants in inoculation methods. The differences in the percentage of teliospore germination may be due to the storage conditions (Quezada-Salinas et al, 2013), probably happened with the sample used as inoculum in the present work, which had a drying period in greenhouse for 3 months, while the rest of the samples had only 5 days of drying. However, further research is needed in this line. In the present study, teliospores with a period of drying in greenhouse for 3 months, preserved in plastic bottles with $CaCl_2$ (J.T. Baker and Macron Fine Chemicals® 94%), stored at 22 ± 2 °C and germinated in PDA medium, and incubated in darkness at 25° C for 24h recorded the above mentioned 45.3% germination.

In the microscopic observation of the sori, it was determined that they consist of masses of teliospores of 63-82 µm (Figure 1A). The teliospores were globose to subglobose, some oviodes or irregular, mainly of brown color. The size of the teliospores varied between 10-12 x 11-13.5 µm, with ornamentation (Figure 1B). Also, irregularly clustered sterile cells of light brown to yellow color or hyalines of 9-16 µm were observed, generally flattened on the contact surface (Figure 1C). These characteristics correspond to the morphological characterization made by Vánky (2012). The germinated

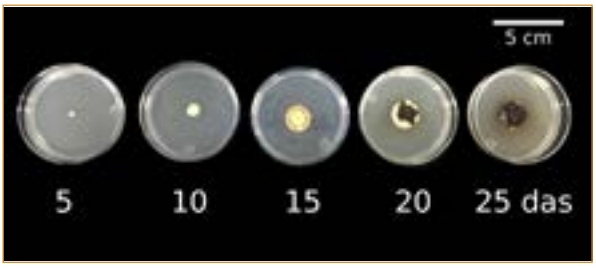

Figure 2. Colonies of *S. reilianum f. sp. zeae* in PDA.
das = days after sowing.

teliospores formed a four-cell septated basidium, each of which formed a basidiospore (Figure 1D), from where numerous secondary sporidia were developed, which later in PDA medium, formed cream yeast-like colonies which 25 days took a light brown to black color (Figure 2).

DNA from monobasidial cultures obtained from the sample used as inoculum, as well as teliospore DNA recovered at the end of the greenhouse experiment, using the pair of primers ITS1F and NL4, amplified a fragment of approximately 1.300 base pairs (Figure 3), corresponded to the expected size of the complete ITS region, plus the portion of the LSU rDNA region of the genome of *S. reilianum* (Stoll et al, 2005).

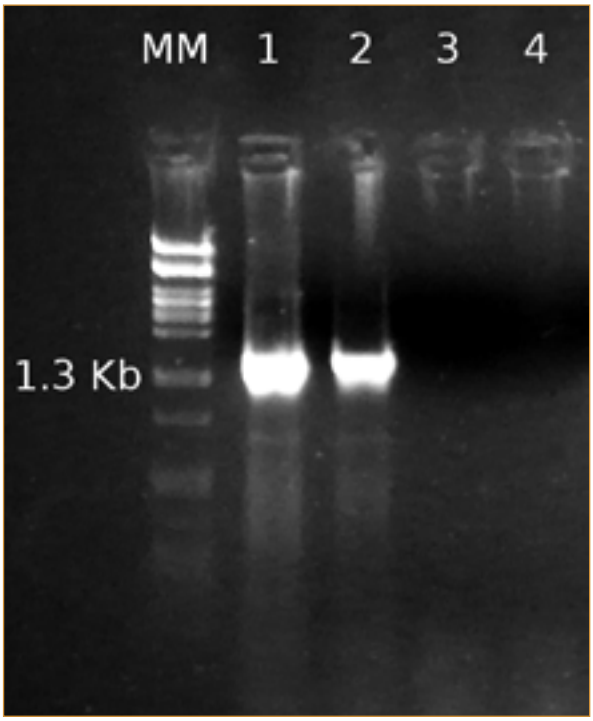

Figure 3. PCR amplification products using primers ITS1F and NL4. MM. Pst1 molecular marker. 1. Monobasidial culture DNA generated from teliospores of *S. reilianum f. sp. zeae* used in inoculation of maize seeds. 2. Monobasidial culture DNA of teliospores of S. reilianum f. sp. zeae collected at the final evaluation in greenhouse. 3. DNA from healthy tassel. 4. Check (water).

The sequences obtained (KY856895, KY856896) have 99% identity to the sequences deposited in GenBank by Schirawski et al. (2008) who sequenced the complete genome of *S. reilianum*, thereby corroborating the identity of the pathogen inoculated and recovered after the greenhouse evaluation. Amplification was not obtained with DNA from the healthy plant tissue and sterile water for PCR.

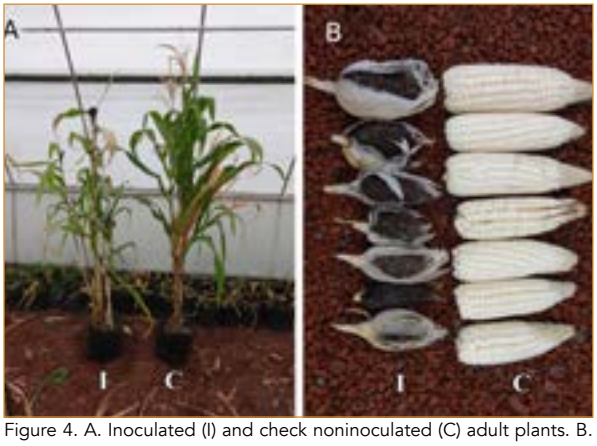

Figure 4. A. Inoculated (I) and check noninoculated (C) adult plants. B. Inoculated (I) and check noninoculated (C) ears.

Evaluation of treatments in greenhouse

The greenhouse experiment planted in the summer cycle (June-October, 2016) was carried out at a mean daily temperature of 27.7 °C, while the average relative humidity was 74.3%. Previously determined none of the materials used as adherent for the inoculation of teliospores had a negative effect on teliospore germination. The percentage of germination observed in the control was 45.3% while in the rest of the treatments the percentages of germination varied in proportions < 1%.

No signs or symptoms of the disease were observed 45 days after flowering in the check plants, whereas plants from inoculated seed showed, the formation of teliospore-filled sori, replacing totally or partially the tassel and ear of the plants (Figure 4), as described by Matyac y Kommedahl (1985b).

The physiological and morphological changes observed in plants of the three hybrids inoculated with the pathogen caused a reduction in the production of pollen and grain, as pointed out by Ghareeb et al, (2011).

The variance analysis did not show statistical differences between the response to infection among the three hybrids evaluated. However, significant differences were observed between treatments (Table 1). Hybrids AS1503, Az 41801 and Euros presented the highest percentage of infected plants when seeds were

Table 1: Percent of maize seedlings infected with S. reilianum f. sp. zeae, generated from seeds inoculated with teliospores using different treatments.

Treatment	Average infection of maize seedlings (%)			
	As1503	Euros	Az 41801	mean
1) Control	0.0	0.0	0.0	0.0 E
2) NaCMC	35.9	35.8	50.0	40.59 CD
3) ADH	5.6	20.8	29.0	18.46 DE
4) Sodium alginate	11.1	20.0	21.4	17.51 DE
5) Brown sugar	4.2	42.1	49.2	31.81CD
6) Agar	41.7	54.2	59.5	51.79 BC
7) Grenetin	86.1	95.2	96.3	92.54 A
9) Inoculum:soil	68.1	72.0	84.1	74.71 AB

Means with the same letter are not significantly different LSD (5%): 29.93, , CV(%): 48.65

inoculated using grenetin as adherent. This treatment induced averages of infected plants of 86.1, 96.3 and 95.2% respectively, demonstrating the efficiency of this method. The second group consisted of treatment 8 (inoculum/soil) and 6 (agar) with percentages of infected plants of 74.71 and 51.79, respectively. The treatment 2 (NaCMC) induced only 40.59% of infected plants and the remaining treatments promoted lower percentages. Noninoculated checks did not show infected plants.

Validation of the best method in field conditions

The field experiment planted in the spring-summer cycle (March-September, 2016) was carried out at a mean daily temperature of 12.7 °C, average precipitation of 31mm, and average relative humidity 62%. Artificial inoculation with grenetin as adherent in the 160 C2-S3 lines of white endosperm maize resulted in a percentage of diseased plants with a maximum of 54.55 in the most susceptible line, while in the AZ 41801 inoculated hybrid resulted in 70.7% infection.

The distribution of the disease in the 160 lines inoculated with teliospores of *S. reilianum* f. sp. zeae using as adherent 10% grenetin was: 84 lines (52.5 %) did not show infection, 29 (18.1 %) with incidence of 1-10%, 27 (16.8 %) with incidence of 11-20 %, 15 (9.4 %) with incidence between 31-55%. The control did not show the disease (Figure5). The 84 C2-S3 lines will be recombined by controlled pollination to generate new cycle of recombination and continue selecting for disease-resistant lines, considering they have good agronomic traits.

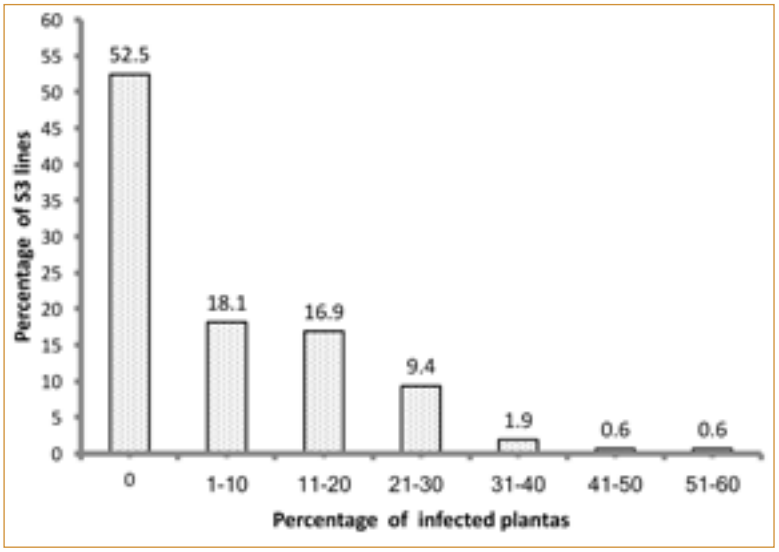

Figure 5. Frequency distribution of white endosperm S3 maize lines with head smut symptoms 45 days after flowering.

The percentages of infected plants were lower than those in the hybrids evaluated in greenhouse conditions, as the C2-S3 lines had 2 previous cycles of disease selection under natural infection conditions.

The use of grenetin as adherent in the inoculation of maize seeds ensures that the inoculum remains in the seed, since the loss of inoculum during transportation and field handling is lower than that obtained with the other methods evaluated. The percentages of infected plants obtained in the present investigation, suggest the use of grenetin as an adherent in the inoculation of maize seeds with teliospores of *S. reilianum*

f. sp. zeae for the evaluation of maize germplasm in a breeding program for selection of genetic resistance to the head smut of maize

The method of inoculation with grenetin proved to be better than the rest of the previously described methods. Field validation of the grenetin inoculation method demonstrated that this method is effective for germplasm selection, since it allows a distinction between resistant and susceptible germplasm under field conditions.

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