Revista Ciência Agronômica, v. 47, n. 2, p. 393-400, abr-jun, 2016
Centro de Ciências Agrárias - Universidade Federal do Ceará, Fortaleza, CE
www.ccarevista.ufc.br

Artigo Científico
ISSN 1806-6690

Transmission of *Stenocarpella maydis* by maize seeds[1]

Transmissão de *Stenocarpella maydis* a partir de sementes de milho

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ABSTRACT - *Stenocarpella maydis* is one of the main fungi associated with maize seeds, being a causative agent of stalk and ear rot, a disease which causes considerable losses for crop-producing regions in Brazil. The organism is considered to be a pest, subject to sanitary standardisation in current programs of seed certification in the country. The aim of this study was to evaluate the transmission rate of the fungus from infected maize seeds. Seeds were inoculated with two isolates using a method of physiological conditioning, in which the seeds are kept in contact with colonies of the fungus for 24 (P1), 48 (P2), 72 (P3) and 96 (P4) hours. Two cultivars were used, one susceptible (C1) and one moderately resistant (C2), and the trial carried out at two temperatures (20 °C and 25 °C). The inoculated seeds were distributed individually into plastic cups containing substrate. The plants were evaluated daily for stand and the appearance of post-emergent symptoms. Based on the number of dead seeds, transmission rates reached a maximum of 90.5% at the P4 inoculum potential, this rate being greater than transmission rates achieved for symptomatic and asymptomatic infection in emerged plants. For the total transmission rate, transmission of the pathogen was seen at all inoculum potentials; these values varying from 25% for cultivar C2 at potential P1 and a temperature of 20 °C, to 93% for cultivar C2 at potential P3 and a temperature of 25 °C.

Key words: Phytopathology. Seed pathology. Water restriction. Stalk and ear rot. Fungus.

RESUMO - *Stenocarpella maydis* é um dos principais fungos que se associam às sementes de milho, sendo um dos agentes causais da podridão do colmo e da espiga, que ocasiona perdas consideráveis em regiões produtoras desta cultura no Brasil. O referido organismo tem sido enquadrado como uma praga sujeita a padronização sanitária em programas de certificação de sementes vigentes no país. O objetivo nesse trabalho foi avaliar a taxa de transmissão do referido fungo a partir de sementes de milho infectadas. As sementes foram inoculadas com dois isolados pelo método de condicionamento fisiológico no qual as sementes são mantidas em contato com colônias do fungo por 24 (P1), 48 (P2), 72 (P3) e 96 (P4) horas. Foram utilizadas duas cultivares, uma suscetível (C1) e outra moderadamente resistente (C2) e o ensaio conduzido em duas temperaturas (20 e 25 °C). As sementes inoculadas foram distribuídas individualmente em copos plásticos contendo substrato. As plantas foram avaliadas diariamente quanto ao estande e aparecimento de sintomas em pós-emergência. Com base em sementes mortas, as taxas de transmissão alcançaram o percentual máximo de 90,5% para o potencial de inóculo P4, sendo esta taxa mais alta do que as alcançadas pelas taxas de transmissão para infecção sintomática e assintomática em plantas emergidas. Em relação à taxa de transmissão total, observou-se a transmissão do patógeno em todos os potenciais de inóculo, variando estes valores entre 25% para C2 no potencial P1 sob temperatura de 20 °C e 93% para C2 no potencial P3 sob temperatura de 25 °C.

Palavras-chave: Fitopatologia. Patologia de sementes. Restrição hídrica. Podridão do colmo e da espiga. Fungo.

DOI: 10.5935/1806-6690.20160047
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[1]Recebido para publicação em 05/02/2013; aprovado em 21/01/2016
Parte da Tese de Doutorado do primeiro autor apresentada ao Programa de Pós-graduação em Agronomia/Fitopatologia do DFP-UFLA; Pesquisa realizada com recursos da CAPES, CNPq e FAPEMIG
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INTRODUCTION

The fungus *Stenocarpella maydis* (Berk.) Sacc. is an important pathogen of the maize crop, causing rotting of the stem, roots and ears, and the death of seedlings (CASA; REIS; ZAMBOLIM, 2006). In Brazil the disease is present in all producing regions, and results in field loss and reduction in grain quality as well as being a potential producer of the mycotoxin diplodiatoxin, which causing death in animals (DORRANCE; HINKELMAN; WARREN, 1998; EDDINS 1930; PINTO, 2006).

The fungus can be found as free mycelium in soil or as dormant mycelium in seeds (FLETT; WEHNER, 1991; FLETT; WEHNER; SMITH, 1992; PINTO; FERNANDES; OLIVEIRA, 1997). Therefore, seeds play a key role in disseminating the pathogen and the transmission to maize plants are an important source of primary inoculum for the disease in question.

The transmission of *S. maydis* through seeds takes on an important role in areas of crops where corn has never been cultivated, or in areas of no-till system. The process of transmission from seeds starts with colonization of the root and base of the stem by the fungal mycelium. This process of colonization may be slow and coincide with the crop cycle (CASA; REIS; ZAMBOLIM, 2006; REIS; CASA; BRESOLIN, 2004).

The colonization and transmission depend on specific characteristics of the host_pathogen interaction. Several factors can influence this process, such as the amount and position of the inoculum in the seeds, the temperature and humidity, the soil microflora, the time of pathogen survival in the seed, and the genotype of the host, among others (ARAUJO *et al*., 2006; MACHADO, 1994; SHAH; BERGSTROM, 2000; TANAKA; MACHADO, 1985).

The interaction between these factors needs to be better investigated aiming generates subsidies to implement sanitary standards (MACHADO; POZZA, 2005).

This work investigated the relationship of inoculum potential and corn genotypes in the transmission of *S.maydis* from seeds to plants.

MATERIAL AND METHODS

Multiplication of isolates and seed profile

Two isolates of *S. maydis*, CML698 and MY2, were used, obtained from the Mycological Collection of Lavras at the Universidade Federal de Lavras, in the State of Minas Gerais, Brazil (MG), and the National Company for Agricultural Reasearch - Embrapa Maize and Sorghum, MG. The isolates were transferred to the Petri dishes and containing a respectively dextrose agar medium (PDA) and kept in a BOD chamber at a temperature of 25 ± 2 °C and a photopetri of 12 hours. Seeds from the maize cultivar RB9308YG, susceptible to *S. maydis* (C1), and RB9108, moderately resistant to *S. maydis* (C2), both from the Riber Seed Company, MG, had previously undergone tests for germination (paper roll) and health (blotter test) according to RAS (BRAZIL, 2009a) and the Manual for Seed Health Analysis [*Manual de Análise Sanitária de Sementes*] (BRAZIL, 2009b).

Seed Inoculation

The technique of osmotic priming was used (MACHADO *et al.*, 2001), in which the seeds are in contact with the pathogen, developed on an agar substrate, for different periods. The seeds were disinfected with sodium hypochlorite (1% active chlorine) for 1 minute and then washed with sterile water. Petri dishes were previously prepared with the PDA culture medium and an added solution of mannitol with the water potential adjusted to -1.4 MPa, according to the SPPM software (MICHEL; RADCLIFFE, 1995). The isolates were transferred to the Petri dishes and incubated at a temperature of 25 °C and a photoperiod of 12 hours, where they remained for five days. The seeds from each cultivar were then evenly placed in a single layer in contact with the fungal colony for 24, 48, 72 and 96 hours, these times corresponding to different inoculum potentials, P1, P2, P3 and P4, respectively. To evaluate the effect of the water restrictor in the absence of the fungus, seeds of both cultivars were deposited on Petri dishes containing the same PDA culture medium modified with mannitol for the same time periods used for inoculation of the fungus.

Evaluation of *S. maydis* transmission in a plant

One seed per plastic cup was sown. The plastic cups with 200 mL were filled with commercial substrate, each treatment with 100 repeatitions equally distributed over four trays. The experiment was conducted in chambers at temperatures of 20 °C and 25 °C ± 2 °C and a photoperiod of 12 hours of light (daylight NSK T10 40W 6500K FL40T10-6 60Hz) and 12 hours of darkness. Plant emergence was evaluated daily, and plants showing symptomatic infection were aseptically placed onto Petri dishes containing the PDA medium and incubated to confirm the presence of *S. maydis* in the tissue. At 28 days after sowing (DAS), the remaining plants were collected and fragments 2 cm long were cut from the base (B) and the last leaf insertion (LI) for analysis. The samples were disinfested in 70% alcohol, sodium hypochlorite (1% active chlorine) and

sterile distilled water for 1 minute and dried on sterilised filter paper. The fragments were placed onto Petri dishes containing the PDA medium and incubated at 25 °C with a photoperiod of 12 hours. After 7 and 15 days, the fragments were individually evaluated under a stereoscopic microscope, in order to observe the characteristic structures of *S. maydis* (MARIO; REIS, 2001).

The detection of *S. maydis* in at least one of the examined fragments per plant was enough to confirm the transmission of *S. maydis* from the seed to the plant.

Experimental design

The experimental design was of randomised blocks in a triple factorial 2 x 2 x 4 scheme (2 temperatures, 2 cultivars, 4 inoculum potentials) with four replications per treatment.

Detection of S.maydis in asymptomatic plants by PCR

Maximum of twenty percent of the plants in each treatment that did not display symptoms of disease were randomly collected and subjected to analysis by conventional PCR. To do this, 2 cm fragments of the plant, taken from the base (B) and the last leaf insertion (LI), were individually macerated in a crucible with liquid nitrogen. DNA was extracted from the macerated samples using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) following the protocol recommended by the manufacturer. The P1/P2 primers described by Xia and Achar (2001), specific for the genus *Stenocarpella*, were used to detect the presence of the fungus in the tissue. Amplification was carried out with 25 µL of the reagent containing PCR buffer (buffer IB - Phoneutria, Brazil - 500 mM KCl; 100 mM Tris-HCl pH 8.4, 1% Triton X-100; $MgCl_2$), dNTPs (2.5 mM of each dNTP), primers (10 mM of each sense or antisense primer) and 5-u-1 µL *Taq* DNA polymerase (Phoneutria, Brazil), with 2 µL of DNA added to make up the total volume. The initial cycle comprised 95 °C for 3 minutes, denaturation at 94 °C for 30 seconds, annealing at 60 °C for 1 minute and extension at 72 °C for 1 minute, with a final extension at 72 °C for 10 minutes, making up a total of 30 cycles. An aliquot of 10 µL was used to separate the PCR products in 1% agarose gel in a TBE buffer, stained with GelRed®, at 150 V for approximately 2 hours. The PCR products were observed in an L-Pix HE model UV transilluminator, (Loccus Biotechnologia, Brazil).

Statistical analysis

The statistical analysis was carried out using the Sisvar® 5.3 software (FERREIRA, 2011). Analysis of variance was performed individually for each isolate of *S. maydis*, as well as for the control (non-inoculated), in the triple factorial 2 x 2 x 4 scheme (2 temperatures, 2 cultivars; 4 inoculum potentials). For the variables, pre-emergent death and rates of transmission, with observations made of symptomatic and asymptomatic plants, the analysis of variance was corrected using the square root transformation of (data+1), as the data contained many values equal to zero. Mean values between treatments were compared by regression, Tukey's test or Student's t-test ($p \leq 0.05$). For the total rates of transmission, all percentages for the rates of transmission of symptomatic and asymptomatic infection, and pre-emergent death, were considered.

RESULTS AND DISCUSSION

Through health tests on the profiles of the used seed batches, the incidence of 28.5% *Fusarium verticillioides* (Sacc.) Nirenberg and 13% *Penicillium* sp. was seen for cultivar C1 (RB9308YG), and an incidence of 25.5% and 11% of the respective fungi for C2 (RB9108). The seeds used in the experiments did not present *S. maydis*. Germination percentages were 98% of normal seedlings for C1 and 96% for C2.

The analysis of variance for pre-emergent seed death and rates of transmission from seeds to plants when the seeds were inoculated with the two isolates of *S. maydis*, revealed a non-significant triple interaction

One of the consequences caused by *S. maydis* is the death of pre-emergent seeds; this death is also often characterised as a confirmation of fungal infection in seeds that were either in direct contact with the pathogen, or indirect contact through transmission from the mother plant. In this case, to estimate pre-emergent seed death for each treatment, the influence of mannitol in the control treatments was observed. The effect of the water restrictor was then subtracted from the treatments with fungus, thus eliminating any possible repressive influence of the restrictor may have on physiological seed quality (COSTA *et al*., 2003).

From Figure 1, it was possible to see that there was a variation in the number of dead pre-emergent seeds, considering the two cultivars and evaluated temperatures. On average, 50% of the planted seeds emerged when they were exposed to the lowest inoculum potential. As the exposure time was increased, the number of dead seeds due to the *S. maydis* fungus also increased, independent of cultivar or temperature. The greatest number of dead seeds was observed with isolate CML698 at the highest inoculum potential (seeds exposed to the pathogen for 96 hours) for cultivar C1 at 20 °C. In this case only 9.5% of the seeds were able to emerge and form normal plants. For seeds of the cultivar C2, infected with the MY2 isolate (Figure 1B), there was greater variation between the different inoculum potentials. For potential P1, there was

a lower rate of dead seeds at 20 °C, despite there being an increase in this index with the increase in inoculum potential of the pathogen, as happened with the CML698 isolate. For cultivar C1, considered susceptible to *S. maydis*, the emergence of plants for P1 was around 65% at temperatures of 20 °C and 25 °C.

The greater number of dead seeds from cultivar C1 for the highest inoculum potential of *S. maydis*, confirms the genetic characteristics of the material, which is considered more susceptible to the pathogen, independent of the temperature at which the work was developed.

The fungus *S. maydis* can colonise the seed embryo and consequently cause pre-emergent death. In cases where the pathogen colonises other parts of the seed, emergence of the plant is not always prevented, however the vigour of the emerged plants may be compromised (CASA; REIS; ZAMBOLIM, 2006; REIS; CASA, 2001). In both cases, planting contaminated and/or infected seeds is an effective way of introducing this agent of stalk and ear rot into fields of maize crops.

The importance of the use of maize seeds infected by *S. maydis* becomes even more worrying in direct-seeding systems, where the availability of substrates and their slow decomposition favour the sporulation, release and spread of the organism in the areas of production. This is considered a major factor in the increased intensity of stalk and ear rot seen in maize grown in a monocrop system (CASA; REIS; ZAMBOLIM, 2003; REIS; CASA, 2001).

The transmission of *S. maydis* from seeds to emerged symptomatic and asymptomatic plants was found in this study, with the presence of the fungus in the tissue of these plants being confirmed by isolations carried out in the laboratory (Figure 2). Based on these observations it was found that the rates of fungus transmission were lower when compared to pre-emergent death. In general, for the lower inoculum potentials P1 and P2, the highest rate of transmission was 2.39% in plants with symptomatic infection, and 3.38% for P1 in plants with asymptomatic infection. In plants with symptomatic infection, despite the presence of pycnidia being common in the field (CASA; REIS; ZAMBOLIM, 2006; CASELA; FERREIRA, PINTO, 2006; REIS; CASA; BRESOLIN, 2004), such was not seen in this study, probably due to plant evaluation being carried out when the plants were still young. However, one symptom seen in this work, and cited as important by Casa, Reis and Zambolim (2006), were the withered leaves of infected plants, turning grey-green and dry, similar to the damage caused by frost.

In this study, differences were seen between transmission rates for plants with symptomatic and asymptomatic infection in relation to the temperature of cultivation. At 25 °C, which is favourable to the development of *S. maydis*, higher rates of transmission of the pathogen were found. For the factor genotype, cultivar C2 (RB9108) displayed a slightly higher resistance to the pathogen than cultivar C1 (RB9308YG), confirming the genetic characteristic of this material as moderately resistant to *S. maydis*. The rates of transmission of seeds with the MY2 isolate were higher than those with the CML698 isolate.

In general, the rate of transmission in plants with symptomatic and asymptomatic infection varied between 3.38%, and 1%, considering varying degrees of genetic resistance by the host, the temperatures and the aggressiveness of the fungal isolates. Although these values are considered low in connection with

Figure 1 - Percentage of pre-emergent seed death in maize caused by the fungus *S. maydis* for two isolates (CML698 and MY2), different inoculum potentials, two temperatures (20 °C and 25 °C) and two cultivars (C1 -RB9308YG and C2-RB9108)

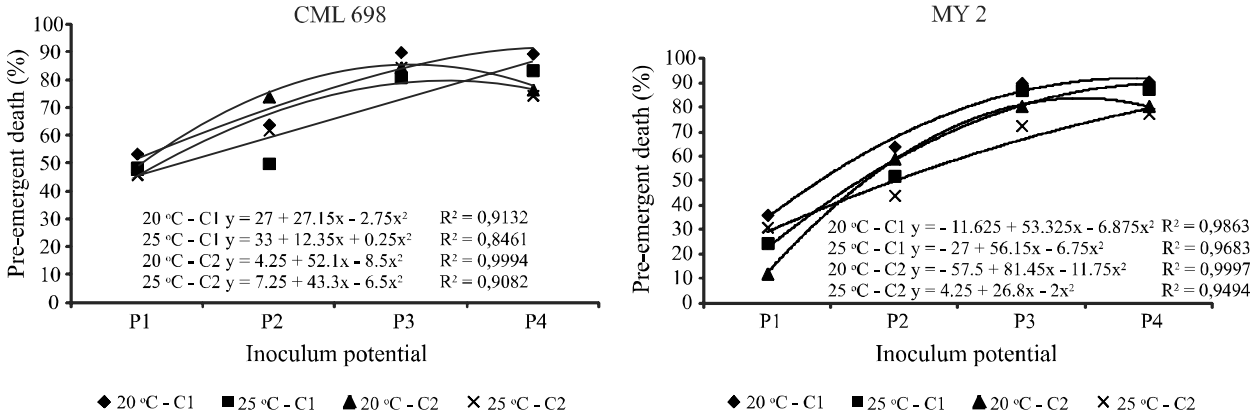
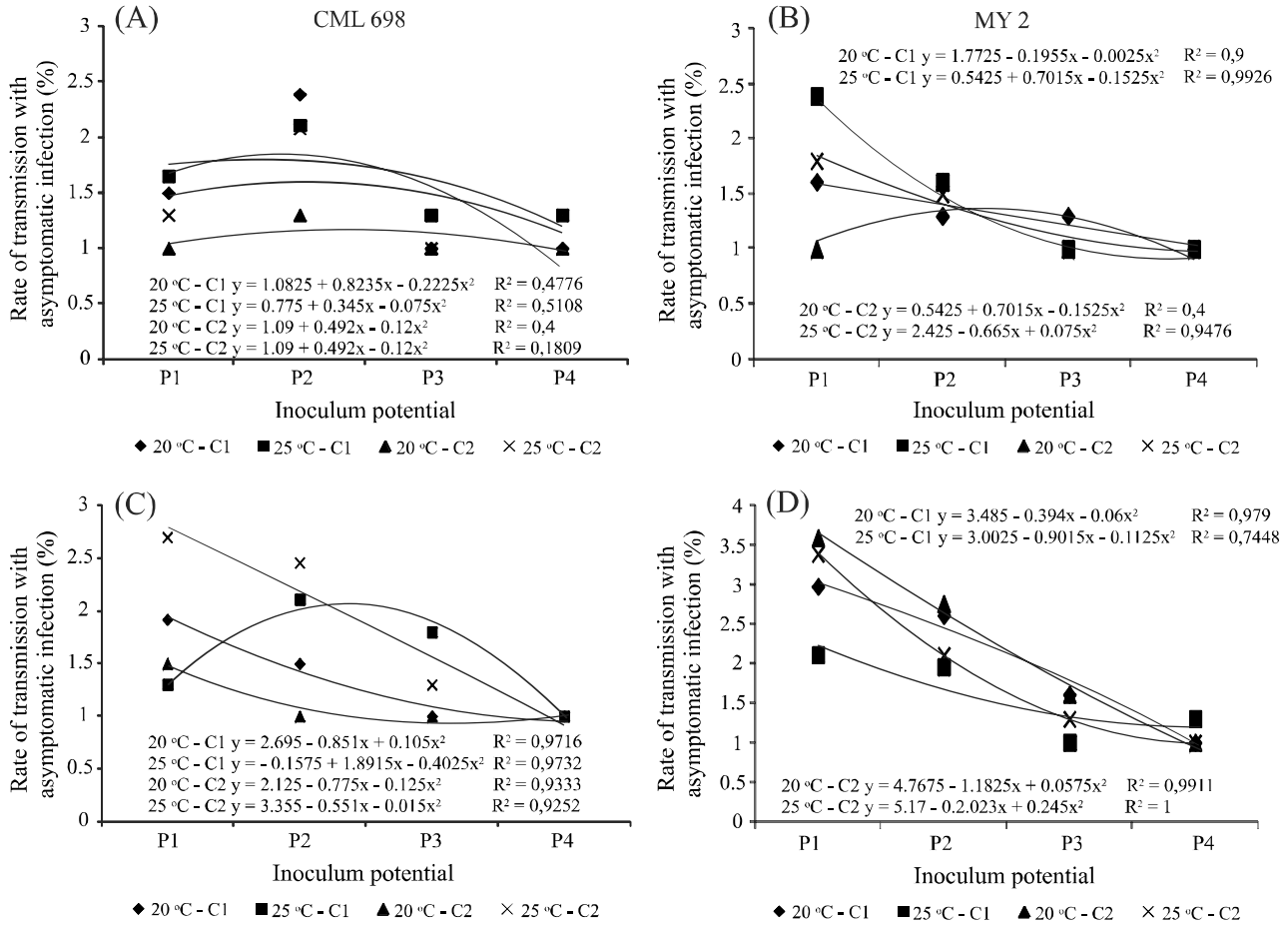


Figure 2 - Transmission rates for *S. maydis* from seed to progeny with and without the induction of symptoms. A and C - transmission of *S. maydis* in seeds inoculated with the CML698 isolate; B and D - transmission of *S. maydis* in seeds inoculated with the MY2 isolate

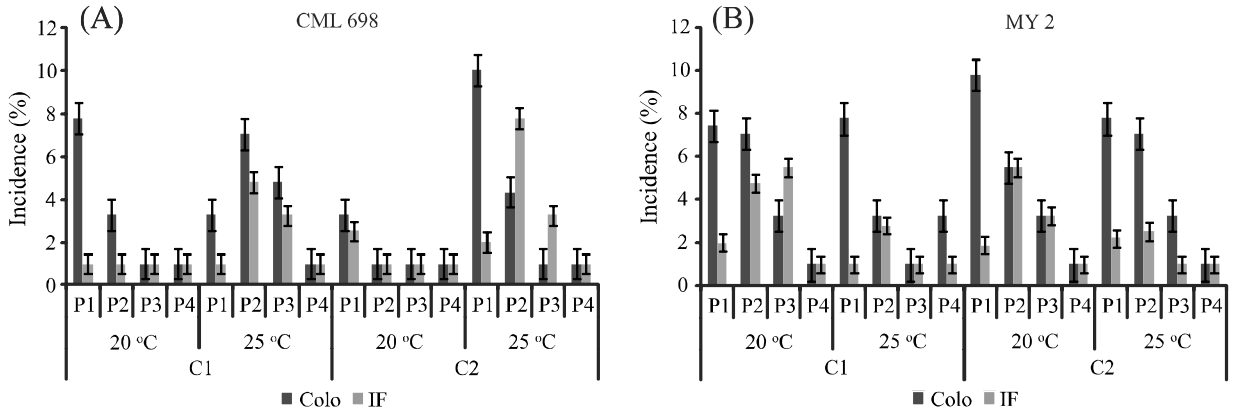


transmission of the fungus from seeds to plants, it is important to point out that the infected plant may also serve as a source of pathogen inoculum in the field for new plants in the same area, and/or ensure its survival under unfavourable conditions, thereby becoming an important source of the inoculum.

In relation to the rate of transmission of *S. maydis* from seeds to plants with asymptomatic infection, considering the position on the analysed plant, the base (B) and the region of the last leaf insertion (LI), a variation was found between values, without there being a standard for this type of association (Figure 3). As already mentioned, the inoculum potentials of P1 and P2 displayed a larger number of asymptomatic plants. The appearance of *S. maydis*, whether in the basal region (B) or at the leaf insertion (LI), may represent the various paths that the fungus can take from the seed to the tissue of the emerged plants. These paths may vary, depending on which part of the seed is contaminated and/or infected, the manner of seed germination, and the time the seed was exposed to the pathogen (inoculum potential), among other factors. For artificial infestation through the technique of priming, the seeds are placed on the fungal colony, which may facilitate infection of the seed coat and endosperm, with infection of the embryo possibly occurring at the highest potentials. Even if seed contamination only occurs at the time of emergence, release of the seed coat may represent an important start to the process of transmission of the disease from seed to plant, both due to the proximity of the inoculum to the plant tissue and the susceptibility of the seedling.

Another important point that deserves attention is transmission of the fungus in plants with asymptomatic infection, where the pathogen is harboured in a quiescent state, thus leading to the development of infection at later growth stages of the maize, which are more favourable to the pathogen, as happens in the two to three weeks after pollination in a humid climate and

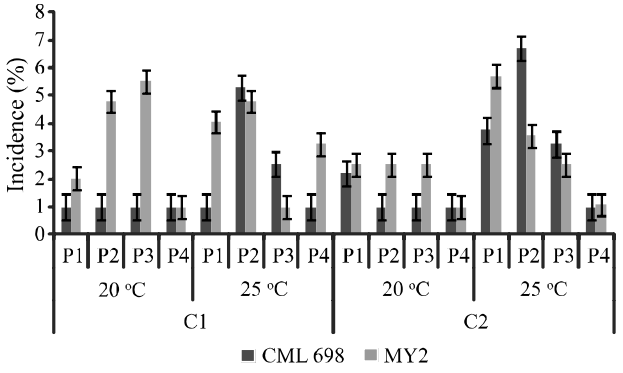
Figure 3 - Incidence of *S. maydis* in plants with asymptomatic infection, sectioned at the base (B) and leaf insertion (LI). A- CML698 isolate and B- MY2 isolate



at temperatures of 28 °C to 30 °C (CASA *et al*., 2007; SHURTLEFF, 1992).

In the evaluations made by using the technique of PCR to detect the presence of *S. maydis* in the fragments from emerged asymptomatic plants at all inoculum potentials (Figure 4), it was found that the highest incidence of the fungus was 6.72% for cultivar C2 with the CML698 isolate, at potential P2 and a temperature of 25 °C, despite the greater number of positive results with higher mean values having occurred with the MY2 isolate. The experiments conducted at 25 °C, in both cultivars and with both isolates displayed the highest incidence.

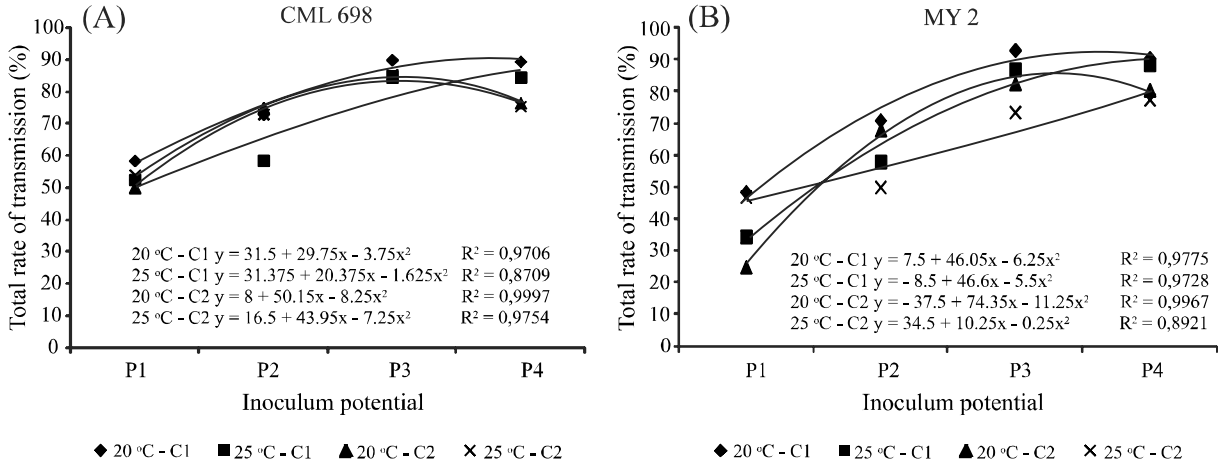
Figure 4 - Detection of *S. maydis* through the technique of PCR carried out on sections of plants with asymptomatic infection



It is important to point out that the technique of PCR was efficient in detecting the pathogen in samples where the traditional isolation test in a culture medium was not effective enough for detection in plants with asymptomatic infection. With these techniques it is possible to detect the presence of the organisms even through external signs, unlike other techniques that only reveal the presence of these agents when in full biological activity. However, a positive result revealed by molecular techniques such as PCR may lead to false positive results, with the detection of nonviable organisms. For the detection of *Stenocarpella* associated with maize seed, this technique has proved promising (BARROCAS, 2008).

From the point of view of epidemiology, the rate of transmission of the pathogen from seed to progeny must include in the final calculation the sum of dead seeds due to the action of the pathogen, and of emerged plants with proven infection by the pathogen (MACHADO, 1994). In that case, for *S. maydis*, the highest values for these rates were seen at the highest potentials, P3 and P4, reaching a maximum of 93% (Figure 5). At the lowest potentials, P1 and P2, the rate fluctuated around 50%. The minimum rate achieved for all treatments was 25% for P1 at a temperature of 20 °C with cultivar C2 (RB9108). For the CML698 isolate, the highest percentages for rate of transmission at potentials P3 and P4 were 90% and 89.5%, and for the MY2 isolate, 93% and 90.5% respectively, relating to the temperature of 20 °C and C1 (RB9308YG). In a study conducted by Siqueira *et al.* (2014), evaluating *Stenocarpella macrospora*, the highest total rate of transmission observed was 85.8%, which occurred in plants from seeds inoculated for 96 hours and grown at a temperature of 20 °C. These results indicate that if the seed is infected, transmission will take place at least through symptomatic plants and/or asymptomatic plants, as also seen in a study with *F. verticillioides* in maize (WILKE *et al*., 2007).

Figure 5 - Evaluation of the total rate of transmission relative to the total of pre-emergent dead seeds and plants with symptomatic or asymptomatic infection. A- CML698 isolate and B- MY2 isolate



Based on these results, the transmission of *S. maydis* from infected seeds to the plant can occur to a varying degree according to the inoculum potential of the pathogen in the seeds, as well as factors such as the aggressiveness of the fungus, prevailing temperatures at the time of germination, and the degree of resistance of the host. This study also reveals that asymptomatic plants from inoculated seeds may harbour pathogen inoculum in their tissues. When establishing the rates of pathogen transmission in general, this fact makes it necessary to take into account this contingent of plants that should apparently be free of infection by pathogens.

Also of paramount importance in this work is the opening that is created for further study, taking into account the role of dead seeds through the action of *S. maydis*, and that remain in the soil throughout the cultivation of the maize.

CONCLUSIONS

1. Pre-emergent seed death is one of the most serious consequences of the transmission of *S. maydis*;
2. The lowest inoculum potentials, P1 and P2, allow most plants to develop, observing the lowest rates of transmission;
3. Asymptomatic plants originated from infected seeds may host the pathogen;
4. The rate of transmission of *S. maydis* by maize seeds was determined as having a minimum of 25% and a maximum of 90.5%.

ACKNOWLEDGEMENT

The authors wish to thank CNPq, FAPEMIG and CAPES for financial support of this work.

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