Inoculum potential of *Stenocarpella* complex and its relation with physiological quality of corn seeds

Potencial de inóculo do complexo *Stenocarpella* e sua relação com a qualidade fisiológica de sementes de milho

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Abstract

Damage caused by *Stenocarpella* complex greatly reduce corn yield. This work was carried out to evaluate the relation between corn seeds physiological quality of a susceptible genotype and a tolerant genotype submitted to different *S. maydis* and *S. macrospora* inoculum potential, separately. Fungal colonies were grown on BDA plus mannitol culture medium, with water potential adjusted at -1.4 MPa. Seeds were inoculated for 0, 24, 48, and 72 hours. Evaluations were carried through seed health test, germination, germination first count, seedling emergence, speed of emergence index (SEI), cold test without media, and esterase enzyme expression. The highest incidence of *Stenocarpella* sp. was observed in the susceptible genotype. The tolerant genotype did not show typical structures of *Stenocarpella* complex at seven days. The increase of inoculum potential of *S. maydis* and *S. macrospora* on corn seeds generate physiological quality reduction. This quality reduction can be observed from 24 hours of inoculation for the susceptible genotype. The tolerant genotype was not physiologically affected, regardless the inoculation time to which they were submitted. The esterase analyses helped to differ *S. maydis* and *S. macrospora* inoculates and can also differ corn seed genotypes, however the reduction of seed quality observed with inoculation of the fungus cannot be detected only by viewing the bands in the gel.

Additional keywords: Diplodia rot; infection; *Zea mays* L.

Resumo

Danos causados pelo complexo fúngico *Stenocarpella* reduzem sobremaneira a produtividade na cultura do milho. Objetivou-se avaliar a relação entre a qualidade fisiológica de sementes de milho de um genótipo suscetível e outro tolerante ao fungo, submetidos a diferentes potenciais de inóculo de *S. maydis* e *S. macrospora*, separadamente. As colônias fúngicas foram desenvolvidas em meio BDA, contendo manitol no potencial hídrico -1.4 MPa, e as sementes permaneceram nesse substrato por diferentes períodos de contaminação (0; 24; 48 e 72 horas). Foram realizadas as avaliações por meio do teste de sanidade, teste de germinação, primeira contagem de germinação, emergência de plântulas, índice de velocidade de emergência (IVE), teste de frio sem solo e expressão da enzima esterase. A maior incidência de *Stenocarpella* sp. foi observada no genótipo suscetível ao fungo. O genótipo tolerante não apresentou estruturas típicas do complexo *Stenocarpella* aos sete dias. O aumento do potencial de inóculo dos fungos *S. maydis* e *S. macrospora* nas sementes de milho gera uma redução da qualidade fisiológica a partir do potencial de inoculação de 24 horas para o genótipo suscetível. Para o genótipo tolerante, os potenciais de até 72 horas não afetaram a qualidade fisiológica das sementes. A análise eletroforetética de esterase pode auxiliar na diferenciação de isolados de *S. maydis* e *S. macrospora*, e de genótipos de sementes de milho; entretanto, a redução da qualidade fisiológica das sementes observada com a inoculação dos fungos não pode ser detectada apenas pela visualização das bandas no gel.

Palavras-chave adicionais: infecção; podridão de Diplodia; *Zea mays* L.
Introduction

The culture of corn has great importance due to its extension of cultivated areas, uses and its annual production, in world agricultural scenario. In Brazil, for the crop 2014/2015, 78-million tons of corn production is estimated. (CONAB, 2015). Factors such as employment in human and animal consumption, and product price, characterize increased demand for hybrid corn seeds with high quality.

In the last years, the number of corn crops under no-tillage system has increased. In addition to the advantages related to the physical maintenance and soil microbiota, increased humidity can predispose plants to attack by microorganisms, one of the factors that directly affect the physiological and sanitary quality in production of seeds. The field fungi require high humidity to grow, as the Stenocarpella complex, Diplodia complex scientific name, consisted of two species, Stenocarpella maydis (Berk.) Sutton, and Stenocarpella macrospora (Earle) Sutton (Brunelli et al., 2005). Currently, the two Stenocarpella species can be detected in all corn producing areas in the country, causing symptoms of diplodia stalk rot, white ear rot and macrospora stain on leaves (Casa et al., 2006).

The ear diseases cause considerable damage in production, particularly in rainy periods, that reduces the quality of the seeds and increases the presence of harmful toxins to human and animal health. Siqueira et al. (2014), studying S. maydis effects in the early stages of corn seedling development and in seeds at 24, 48, 72 and 96 hours inoculum potentials, concluded that the greater exposure time of seeds to fungal colony, higher were the negative effects of these organisms, causing reduction in seed vigor, seedling speed of emergence, at the final stand and the initial development of corn seedlings of two cultivars, one susceptible and another moderately tolerant to the pathogen.

The genotypes study with different resistance and susceptibility levels is necessary to elucidate the relation between the pathogen and the available germplasm. Wiser et al. (1960), reported that there is no germplasm with complete resistance to Stenocarpella sp. It was also evident, that genetic variability for resistance to this fungal complex exists, and this suggests that through specific improvement programs, it can be achieved resistant hybrids to white ear rot. Thus, this research aimed at evaluating the relation between the physiological quality of corn seeds of a susceptible genotype and another tolerant to fungi, subjected to different S. maydis and S. macrospora inoculum potentials separately, in order to elucidate the relation of this pathogen with different studied genotypes.

Material and methods

Two genotypes of corn seeds were used, provided by Dow AgroSciences Company, one was classified as susceptible and the other as tolerant to Stenocarpella sp. complex, both genotypes showed 100% of germination (BRASIL, 2009a). Seeds were disinfected with 2% sodium hypochlorite solution for a minute, washed and dried on filter paper at ambient laboratory conditions, and then subjected to the inoculation procedure.

Fungal isolated were obtained at mycological collection of the Seed Pathology Laboratory of the Universidade Federal de Lavras. The one isolated from Stenocarpella maydis, recorded as LAPS 292, was collected in the city of Sete Lagoa - MG, and the isolated from Stenocarpella macrospora, recorded as LAPS 012, was collected in Passo Fundo - RS.

Corn seeds were inoculated with S. maydis and S. macrospora by the water restriction method in agar substrate (Machado et al., 2001). Water potential of -1.4 MPa with addition of mannitol at 2% BDA mean, in Petri plates (15 cm of diameter) was used. Separately, the inoculum of each fungal species was transferred back to the plates by the transfer of colony discs method, next plates were randomly distributed in a 25°C growth chamber with 12 hours photoperiod for seven days. After this period, seeds of susceptible and tolerant genotypes were separately distributed over the S. maydis and S. macrospora colonies and maintained under mycelium for four inoculum potentials: zero, 24, 48 and 72 hours. Just after the exposure at different potentials, seeds were dried for 24 hours in laboratory ambient conditions and subjected to evaluations.

The analysis of seeds sanity was evaluated by “Blotter test” with freezing. Eight replicates of 25 seeds were distributed on Petri plates (15 cm of diameter) were used, containing three filter paper sheets previously sterilized and wetted with distilled water. Seeds were incubated for 24 hours at 20 ± 2°C, in chamber with 12 hours photoperiod and transferred to freezer for another 24 hours and re-incubated at 20 ± 2°C, for five days. Thus, the reading was performed at the 7th day, using a stereoscopic microscope, and results expressed in percentage (BRASIL, 2009b).

For the germination test, the substrate for sowing was the “Germitest” sheet type, moistened with distilled water in amount of 2.5 times of the paper dry weight. Seeds were placed in 25°C germinator and counting carried out at 4th (germination first counting) and at 7th day, after sowing. Evaluations were performed according to the Rules for Seed Testing (BRASIL, 2009a), with four replications of 50 seeds and results expressed as percentage of normal seedlings.

Seedling emergence and speed of emergence index was performed with four replications of 50 seeds. Seeds of each replication were sown in plastic trays containing substracts sand + soil in 2:1 ratio, with 60% of field capacity. Trays with the seeds were placed in a 25 ±C growth chamber, combined with intermittent light and dark (12 hours), and irrigation management was carried out as necessary. The evaluation of normal seedlings was
daily conducted, for the speed of emergence index (SEI) calculation by Maguire formula (1962) until the 10th day after the test installation.

For the cold test four replicates of 50 seeds were distributed on paper substrate (germitest type) moistened with amount of distilled water equivalent to three times dry weight of the substrate were used. Rolls were made as germination test and after sowing, packed in plastic bags and kept in a 10 °C cold chamber (Cicero & Vieira, 1994). The evaluation of germinated seeds was performed at the 7th day after sowing and the result, expressed as percentage of normal seedlings (BRASIL, 2009).

The analysis of esterase isoenzyme was performed by electrophoresis (Alfenas, 1998). For this analysis, the inoculated seeds and isolated from *S. maydis* and *S. macrospora* were macerated in liquid nitrogen and polyvinylpyrrolidone (PVP) presence in mill and stored at -86 °C.

The buffer used to extract the esterase enzyme (EST) was Tris HCL 0.2 M, pH 8 by adding 0.1% mercaptoethanol at 250 uL ratio per 100 mg of seed sample. The same rate of extraction buffer per 15 mg of sample was used for the isolate from *Stenocarpella* sp. The material was homogenized in vortex and kept into a refrigerator overnight, followed by centrifugation at 14,000 rpm for 60 minutes at 4 °C.

Polyacrylamide gel at 7.5% (separating gel) and 4.5% (concentrating gel) was made for the electrophoretic running. The gel/electrode system used was the tris-glicina pH 8.9. An amount of 50 uL sample supernatant was applied in gel, and the electrophoretic running performed at 150 V for five hours.

After running, the gel was revealed for the esterase enzyme (EST) and visualized by UV transilluminator, according to Alfenas (1998).

The data of physiological and vigor tests were statistically analyzed in completely randomized design in a 4 × 2 factorial scheme of four treatments (inoculum potentials - zero, 24, 48 and 72 hours) and two fungal species (*S. maydis* and *S. macrospora*). Data related to the incidence of pathogens were previously transformed into (x + 1)½. Variance analysis was performed using Sisvar® software (Ferreira, 2011), with p<0.05 by the F test. The effect of fungi inoculum potential was studied by analyzing the polynomial regression with the plots using the SigmaPlot software version 12 (SYSTAT Software Inc.).

### Results and discussions

In corn seeds of susceptible genotype were found significant isolated effects from the inoculum potentials of *Stenocarpella* sp. (Figure 1) and fungal species for sanity test.

![Figure 1 - Stenocarpella sp. incidence on Blotter Test first evaluation of fungi susceptible seeds submitted to different inoculum potentials. * Data transformed on (x + 1)².](image)

*Stenocarpella* sp genus needs more time to develop typical structures for detecting in seeds; in this case, seeds even inoculated separately with the two species and subjected to different inoculum potentials, showed no large amount of fungi in the reading at seven days, the *S. maydis* species was detected in larger amount for the susceptible genotype to fungi. The means of incidence data were 2% for *S. maydis* and 0.62% for *S. macrospora*, similar results to those obtained by Siqueira et al. (2014), which reported higher *S. maydis* capacity for infecting corn seeds.

Regarding the effect of inoculum potentials of susceptible genotype seeds, it was found by regression analysis of a linear tendency that, as the inoculum potential was increased, higher the incidence of *Stenocarpella* sp.(Figure 1), demonstrating that these potentials were efficient for inoculation. However, for seeds of tolerant genotype, there was no incidence of the pathogen in any seed in *Blotter
reading performed at seven days, showing the effect of this genetic characteristic on the incidence of *Stenocarpella* sp. in seeds. In Brazil, there are few reports about the genetic resistance of commercial hybrids to *Stenocarpella* species. Mario et al. (2003), observed no significant differences between these hybrids as to their resistance level to both species, questioning the possibility that the resistance to *S. macrospora* and *S. maydis* have distinct genetic controls.

For the susceptible phenotype, there was difference in seeds germination due to the inoculation periods, with linear effect. Lower germination values were observed in seeds as it increases the exposure time of seeds to the inoculum (Figure 2). Which confirms the relation of *Stenocarpella* sp. incidence and decrease in germination, since for the same genotype was verified greater incidence as it enhances the inoculum potential (Figure 1).

**Figure 2** - Normal seedlings percentage of fungi susceptible corn seeds submitted to different inoculum potentials, obtained by germination test (G%), and germination first counting (FC%).

There was no significant difference between *Stenocarpella* sp. species inoculated in seeds for the germination test. Regarding the germination first counting was significant effect for fungi species (Table 1).

**Table 1** - Germination (G%) and germination first counting (FC%) of susceptible corn seeds inoculated with *S. maydis* and *S. macrospora*.

<table>
<thead>
<tr>
<th>Fungi</th>
<th>FC (%)</th>
<th>G (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. macrospora</em></td>
<td>96</td>
<td>87</td>
</tr>
<tr>
<td><em>S. maydis</em></td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>CV (%)</td>
<td>2.03</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* Significant difference by analysis of variance, P <0.05 by F Test.

Susceptible genotype seeds inoculated with *S. macrospora* species had lower germination in the germination first counting, in which seed vigor is evaluated. Latterell & Rossi (1983), comparing the fungi pathogenicity of *Stenocarpella* sp. genus, found that *S. macrospora* is more aggressive than *S. maydis* during the initial stages of plant development, which can affect vigor evaluations in the newly germinated seedlings.

The influence of inoculum potentials on the germination first counting was linear and with negative effects, as it has also been verified for germination (Figure 2). These results are similar to those found by Grandis et al. (2008), who studied the severity of leaf stain of diplodia (*S. macrospora*) and its relation with pathogen and germination incidence, and concluded that the correlation between *S. macrospora* incidence in the grains and germination percentage was high and negative, showing that this pathogen affects grain germination or corn seeds. The presence of pathogens in seeds is associated with decreased germination power, according to Muniz et al. (2004). Casa et al. (2006), studying the damage caused by *S. maydis* and *S. macrospora* in corn crop, noted the association of these fungi to the decrease in the germination percentage, indicating the attack to the seed embryo, and in the cases where seeds germinated, seedling vigor has been compromised. On the other hand, Mendes et al. (2012) evaluated the behavior of corn hybrids inoculated with fungi that causes the damaged grains complex, and for the inoculation with *S. macrospora* was significant difference for grain yield, and some studied commercial hybrids are considered as resistant and others as susceptible, according to the seed producing companies.

For the tolerant genotype, different inoculum potentials and *Stenocarpella* sp. species that seeds
were submitted did not provide significant differences in the normal seedlings percentage in germination tests and germination first counting, in both tests the values observed were high, between 97 and 100%. The incidence of *Stenocarpella* sp. in seeds of this genotype was not observed, which indicates the importance of seeds sanity and their influence on the physiological quality.

For the seeds of susceptible genotype to *Stenocarpella* sp. there was no significant difference for fungi species inoculated on seedling emergence, means of emerged seedlings for seeds inoculated with *S. maydis* were 98%, and for seed inoculated with the isolated from *S. macrospora* were 97%.

Within 24 hours of inoculation with *Stenocarpella* sp. there was no negative effect on seedling emergence of susceptible seeds, in this potential emergency was 100% (Figure 3). There was a reduction in seedling emergence only after the 24 hours potential; for seeds submitted to 48 and 72 hours inoculum potentials, means were 98% and 92%, respectively. Which it indicates, as the germination test, that the presence of these fungi in corn seeds can cause damage related to the physiological quality; however, to affect the seedling emergence is necessary a higher inoculation period of seeds. The pathogen presence on the seeds of the susceptible genotype to fungi delayed seedling emergence, harmful fact in the development and establishment of the initial stand of culture.

![Graph showing the relationship between inoculum potentials and seedling emergence percentage](image.png)

**Figure 3** - Seedling emergence percentage of corn seeds susceptible to *Stenocarpella* sp. submitted to different inoculum potentials.

In the emergence test was observed decreased number of plants emerged from 24 hours potential (Figure 3) due to the exposure to greater potentials for infection, causing slightly progressive damage to the quality of seeds and influencing seedling emergence. However, there was no significant difference between the speed of emergence index means in which evaluates vigor, in susceptible genotype seeds to fungi for anyone of the studied factors.

Even with susceptibility to fungi, the used inoculum potentials were not enough to affect the performance of corn seeds, regarding the seedlings speed of emergence index. They do not differ from each other, with values between 12.22 and 12.19 for *S. maydis* and *S. macrospora*, respectively.

For the seeds of tolerant genotype to *Stenocarpella* complex there was significant effect for fungal species and for the inoculum potentials (Figure 4), separately.

Means for seeds inoculated with *S. maydis* of 12.84 were higher than those observed for *S. macrospora* of 12.55. Machado et al. (2001) evaluating the performance of corn seeds submitted to water restriction in the *S. maydis* presence observed no seed germination.

The behavior of tolerant seeds submitted to different inoculum potentials for the results of speed of emergence index followed a linear trend (Figure 4), with positive effects. The longer the exposure time to the fungi, the greater the speed of emergence index, i.e. seedlings emerged in a shorter time, which confirms that for seeds considered as tolerant, fungi does not affect the physiological quality with the tested inoculation times. For corn seeds of susceptible genotype, inoculation potentials significantly affected seed vigor (Figure 5) in cold test.

From 24 hours of inoculation there was a decrease in the percentage of seeds germination subjected to cold test, being the means observed in the cold test result of 96% for the 48 hours inoculum potential, and 89% for the 72 hours potential; thus, the largest inoculation potentials of *Stenocarpella* sp. damaged seed vigor.
Figure 4 - Corn seedlings speed of emergence index (SEI) tolerant to Stenocarpella sp. submitted to different inoculum potentials.

Figure 5 - Normal corn seedlings percentage susceptible to Stenocarpella sp. submitted to different inoculum potentials, acquired on cold test.

The cold test is used to evaluate the performance of the seeds subjected to low temperatures and high humidity, and when associated to the presence of pathogens, vigor test can be even more sensitive to identify small differences in the physiological potential of seeds (Ramos et al., 2010). This is an unfavorable condition for the germination of corn seeds (low temperature and high humidity) however, it can offer good conditions for the development of some pathogens. Nerbass et al. (2008) by studying the sanity of corn seeds in the state of Santa Catarina and Rio Grande do Sul, where temperatures are usually mild, they observed high \textit{S. maydis} incidence in the evaluated lots.

Tolerant seeds to \textit{Stenocarpella} sp., regardless of inoculation period and fungi species, show no differences in relation to vigor estimated by cold test, with values between 98 and 100%.

In the analysis of the activity esterase isoenzyme (Figure 6) has observed that in tolerant seeds inoculated with \textit{S. maydis}, there was an increasing esterase activity according to the increased inoculum potential that seeds were subjected (Figure 6 - indicated number - 1).

As esterase (EST) is directly connected to the formation of cell membranes, we can relate this result to the pathogen attack affecting the membrane according to the inoculum potential, as in the 48 and 72 hours potentials there was an increased activity of this enzyme (Figure 6 - indicated number 1). For the others materials, it is observed a constant activity of esterase.
Figure 6 - Esterase enzyme activity (EST) in susceptible and tolerant corn seeds inoculated with S. maydis (MY) and S. macrospora (MC) submitted to different inoculum potentials.

For fungal isolated, an isoform may be characteristic of Stenocarpella sp. genus (Figure 6 – number indicated two). This same isoform is present in most inoculated materials at different intensities, very evident in the 72 hours potential in susceptible seeds inoculated with S. maydis.

By esterase enzyme activity it is possible to separate the isolated from S. maydis and S. macrospora, however, it is not possible to separate them in the seeds inoculated with each isolated. Due to the intensity of distinctive bands of cultivars in fungal isolated, it infers that they did not appeared when inoculated in seeds, because in this case, isoenzymes were at insufficient concentration to form bands on gel. It is also evident from the comparison of the electrophoretic profiles where isoenzymes set is different in each genotypes (susceptible and tolerant); however, reduced physiological quality of seeds that was observed with the increase of fungal inoculation time in both genotypes cannot be detected by bands visualizing on gel. Dorrance et al. (1999), studying isoenzymatic polymorphism and cultural variability of S. maydis isolated that were collected in the US and South Africa, concluded that a low level of isoenzymatic polymorphism was detected in this collection of isolated and found only by esterase, hexose kinase and malate dehydrogenase. The color of colonies and pycnidia production were variable along several experiments, indicating that these phenotypes are variable genetic markers, highlighting the difficulty of differentiation and the establishment of a pattern for S. maydis species, thus the isolated even from the same species, still have peculiarities.

Carvalho et al. (1997), though esterase, acid phosphatase and peroxidase profiles distinguished Colletotrichum gossypii-var. Cephalosporioides, with the use of electrophoretic patterns employed as biochemical markers, a useful tool in differentiating of two fungi.

Silva et. al. (1998) studying electrophoretic patterns of isoenzymes in corn coleoptile in association with microorganisms found increased intensity of esterase enzyme in coleoptile obtained from infected seeds with Fusarium moniliforme and isoenzymatic patterns of coleoptile obtained from seeds infected with Fusarium moniliforme, Aspergillus flavus and Penicillium spp. are changed to esterase isoenzyme.

Isoenzymatic patterns are also used to differentiate aspects connected to quality and even to differentiate genetic materials. Gomes et al. (2000), used these patterns to study the effect of heterosis on physiological quality of corn seeds and found that certain crosses produced high quality seeds, relating the results of physiological tests with isoenzymes activity. The difference in the pattern of esterase isoenzyme for tolerant and susceptible seeds to fungi (Figure - number indicated 3) may be a useful tool to differentiate corn genotypes. In the treatments where there is increased activity of this enzyme, there is also a higher membrane disruption.

Conclusions

The highest incidence of Stenocarpella sp. is observed in seeds of the susceptible genotype to fungi. The tolerant genotype has no structures of Stenocarpella sp. complex in reading at the seven days of the Blotter test with inoculum potential of until 72 hours.

There is a reduction of seed quality of the susceptible genotype inoculated with the Stenocarpella sp.
sp. complex from the 24 hours inoculum potential. The inoculum potentials of until 72 hours are not sufficient to affect the physiological quality of seeds of corn genotypes tolerant to *Stenocarpella sp.*

The esterase isoenzyme evaluation may help in the differentiation of isolated *Stenocarpella sp.* and genotypes corn, however, the reduction of physiological quality of seeds observed with the fungi inoculation cannot be detected only by bands visualization on the gel.

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