Colonization of maize seeds by two species of *Stenocarpella* transformed with fluorescent proteins and assessed through scanning electron microscopy[1]

Carolina da Silva Siqueira[2*], José da Cruz Machado[2],
Carla Lima Corrêa[2], Ellen Noly Barrocas[3]

ABSTRACT – *Stenocarpella maydis* and *Stenocarpella macrospora* species causing leaf spots and stem and ear rots, can be transported and disseminated between cultivating areas through seeds. The objective was to transform isolates of species of *Stenocarpella* with GFP and DsRed and to correlate different inoculum potentials with the effect caused by the presence of these pathogens in the tissues of maize seeds. The isolates were transformed with introduction of the genes in their nuclei, employing the technique of protoplast transformation. Seeds were inoculated by osmotic conditioning method with transformed and not transformed isolates, with different periods of exposition of seeds to those isolates, characterizing the inoculum potentials, P1 (24 h), P2 (48 h), P3 (72 h) and P4 (96 h). The seeds inoculated with isolates expressing GFP and DsRed in both species elucidated by means of the intensities of the emitted fluorescence, the ability of those organisms to cause infection and colonization in different inoculum potentials. The potentials P3 and P4 caused the highest levels of emitted fluorescence for the colonization by both pathogens. A comprehensive and abundant mycelial growth in the colonized seed structures were well visualized at potential P3 and P4 by means of SEM.

Index terms: seed pathology, genetic transformation, GFP, DsRed protein, fungus.

Colonização de sementes de milho por duas espécies de *Stenocarpella* transformados com proteínas fluorescentes e avaliadas por microscopia eletrônica de varredura

RESUMO – *Stenocarpella maydis* e *Stenocarpella macrospora*, espécies causadoras de manchas foliares, podridões em plantas e grãos ardidos de milho, podem ser transportadas e dispersas para áreas produtoras através das sementes. O objetivo foi transformar isolados de espécies de *Stenocarpella* com GFP e DsRed e correlacionar diferentes potenciais de inóculo com o efeito causado pela presença desses patógenos nos tecidos de sementes de milho. Os isolados foram transformados por meio da introdução dos genes nos seus núcleos, empregando a técnica de transformação de protoplastos. Sementes foram inoculadas pelo método de condicionamento osmótico com isolados transformados e não transformados, com diferentes tempos de exposição das sementes a estes isolados, caracterizando os potenciais de inóculo, P1 (24 h), P2 (48 h), P3 (72 h) e P4 (96 h). As sementes inoculadas, com isolados transformados expressando GFP e DsRed, de ambas as espécies, elucidaram por meio das intensidades das fluorescências emitidas, a capacidade desses organismos de causarem infecção e colonização em diferentes potenciais de inóculo, tendo P3 e P4, provocado as maiores intensidades de fluorescência pelas colonizações dos patógenos. Um abrangente e abundante crescimento micelial nas estruturas colonizadas das sementes foi observado nos potenciais P3 e P4, por meio da MEV.

Termos para indexação: patologia de sementes, transformação genética, proteína GFP, proteína DsRed, fungo.

Introduction

Many pathogenic fungal species, like those of the so called "*Stenocarpella* complex" may be transmitted by maize seeds. Those species are responsible for rots on the stalk and ear on maize, being also potential producers of toxins noxious to animal health (Petatán-Sagahón et al., 2011) and causing drop on the price of the product to destined as certificated seeds in the market (Ribeiro et al., 2005).

Depending on the environmental conditions, the maize seeds can ensure the survival of some fungal species into the tissues of seed coat for a short storage period. However, the

[1]Submitted on 10/22/2013. Accepted for publication on 04/02/2014.
[2]Departamento de Fitopatologia, - UFLA, Caixa Postal 3037, 37200-000 - Lavras, MG, Brasil.
[3]Department of Plant and Environmental Sciences, 2360- Taastrup, Denmark.
*Corresponding author <kerolpet@gmail.com>

fungi *S. maydis* and *S. macrospora* survive in the internal tissues of seeds (endosperm and embryo) mainly on mycelial form. Thereby, when the seed comes in contact with the soil water and receives the stimuli from the environment to start germination process, the fungal mycelium starts growing from internal tissues to seed's surface. Thus, when growing on the seed the fungus reaches the roots and coleoptiles; and through coleoptiles fungus reach to soil's surface and may cause death of that seedling (Casa et al., 2006).

Thereby, the study of pathogen dynamics, at the internal tissues infection moment, as well as the pathogen location within the seed, becomes of paramount importance for the understanding the host-pathogen relationship. In this sense, some molecular markers, such as the green fluorescent protein (GFP) originating from the jellyfish *Aequorea victoria*, and the red fluorescent protein, originating from the mushroom anemone *Discosoma* sp. (DsRed) used on fluorescence microscopy; besides the scanning electron microscopy (SEM), have been successfully used for the live observation of the fungal structures within the seeds, as well as to clarify the processes of infection and colonization of seed internal tissues by the pathogenic agents (Cristea et al., 2005; March et al., 2003).

Based on the previous information, the aim of this study was to obtain different isolates of the two species of *Stenocarpella*, transformed by molecular markers expressing the GFP and DsRed fluorescent proteins by adjusting already described protocols; as well as correlating different inoculum potentials in the infection and colonization of the seed internal tissues through the fluorescence microscopy and scanning electron microscopy (SEM).

Material and Methods

Characterization of isolates and source of seeds: two different isolates of *Stenocarpella maydis* (Berkeley) Sutton [Syn. *Diplodia maydis* (Berkeley) Saccardo] as well as two different isolates of *Stenocarpella macrospora* (Earle) Sutton [Syn. *Diplodia macrospora* Earle] were used for this study. One of the *S. maydis* isolates, registered as CML698 was obtained at the mycological collection of Federal University of Lavras (UFLA), located at Lavras, state of Minas Gerais (MG); and the other isolate, registered as MY2 was obtained of Embrapa Maize and Sorghum, located at Sete Lagoas, MG. The two isolates of *S. macrospora*, registered as CMLAPS375 and CMLAPS10, respectively were obtained at the mycological collection of the Seed Pathology Laboratory of UFLA. The maize seeds (cv. RB 9308 YG), with 98% germination (Brasil, 2009a), were provided by the company Riber KWS Seeds, based at Patos de Minas, MG. The cv. RB 9308 YG were submitted to health testing which reveled the presence of *Fusarium verticillioides* at level of 28.5% and *Penicillium* sp. at 13% incidence in the seeds (Brasil, 2009b).

Transformation of isolates: both of isolates of *S. maydis* and both of *S. macrospora* were transformed by using the plasmids pSC001 and pSC002, which contain the *hpr* gene, for resistance to antibiotic hygromycin B; as well as the p*ToxA* promoter gene originated from *Aspergillus nidulans* (Maor et al. 1998). Following methodology described by Sambrook and Russell (2001), competent cells of bacterium *Escherichia coli* were used for multiplication of these plasmids; and for transformation of isolates, the protocols previously described by Maier et al. (2005) and Silva et al. (2009) for *Fusarium graminearum* and *Sclerotinia sclerotiorum* were used with some methodological modifications.

For obtaining protoplasts of *S. maydis* and *S. macrospora*, both fungi were grown on PDA culture medium (200 g agar; 20 g dextrose; and 200 g potato) for 5 days, at 25±2 °C and photoperiod of 12/12 h (L/D). After such period, mycelium disc with 1 cm^2 were cut from the growth zone of each colony and transferred to Erlenmeyer flasks with 100 mL capacity, containing 50 mL of liquid potato-dextrose culture medium. For generation of protoplasts, immediately after inoculation the flasks with the mycelial discs were incubated under constant shaking, provided by a mechanized horizontal shaker, set to 75 rpm, where they were maintained, at 28 °C, for 3 h. After fungal growth, mycelial mass of each colony was aseptically filtered and immediately dehydrated with vacuum pump. Afterwards, 100 mg of dry mycelium, from each isolate, was added into a tube with 3 mL of the KCl osmotic stabilizer (0.7M) and 10 g of lysing enzymes [Sigma®-L1412 (lysing enzymes from *Trichoderma harzianum* lyophilized powder)] at a ratio of 10 $mg.mL^{-1}$ osmotic stabilizer.

Only the produced protoplasts were filtered through a previously sterilized cheesecloth layer, and then centrifuged at 600 x g (RCF), at 4 °C, for 5 min. Immediately after, these protoplasts were re-suspended in KCl (0.7 M), at 4 °C, and again re-suspended in storage buffer composed by four parts of the solution STC [0.8 M sorbitol; 50 mM Tris HCl (pH 8.0); and 50 mM CaCl2] and one part of the solution SPTC [0.8 M sorbitol; 40% PEG 4000; 50 mM Tris HCl (pH 8.0); and 50 mM CaCl2]. Subsequently, to the suspensions of final concentration of approximately 10^7 protoplasts. mL^{-1}, were added 10 µl of plasmid DNA, at a ratio ranging between 0.35 and 1.66 $mg.\mu L^{-1}$, which were then kept on ice for 30 min. Afterwards, 1 mL SPTC was added to solution, and kept at room temperature for more 20 min. After such period, protoplast suspensions were separately poured into Erlenmeyer flasks containing 200 mL regeneration medium

[0.1% yeast extract (Sigma-Y4250 - 250 g); 0.1% hydrolyzed casein (Sigma-C8845 - 500 mg); 34.2% sucrose (Sigma-84100 – 1 kg); and 1.0% Agar granulated (Difco-1016141000 - 1 kg)]. Subsequently, this regeneration medium, containing the transformed isolates was poured into Petri dishes (9 cm Ø), and incubated, at 25±2 °C, for 72 h. After this period, to each of the plates was added 10 mL of water-agar medium containing the antibiotic hygromycin-B, at a concentration of 100 $mg.mL^{-1}$, and kept at 25±2 °C, and 12/12 h photoperiod, for 15 days, into an incubation chamber.

After protoplast transformation, mycelium of each of the two species of the fungus, containing the marker genes for proteins GFP and DsRed were separately transferred to PDA culture medium, to which was added the antibiotic hygromycin-B, at concentration of 100 $\mu g.mL^{-1}$, as well as to the Oatmeal-Agar (OA) culture medium (20 g agar, 30 g oatmeal, and 1 L H_2O) also added with hygromycin-B at same concentration used in the PDA medium. All isolates were assessed both by their morphological characteristics as by their cultural characteristics as mycelial coloration and mode of growth of the colonies. For observing stability of the different isolates of transformed fungus, successive transfers were performed on PDA medium containing 100 $\mu g.mL^{-1}$ of antibiotic hygromycin-B. Both for the assessments of bioassays as for stability tests, observations were performed with the aid of a fluorescence stereomicroscope (brand Leica; model M165FC) and of a fluorescence microscope (brand Zeiss, model AxioVision Z.1) equipped with the software AxioVision Imaging System, which possesses special filters for observation of the marker genes for the proteins GPF (filter with a wavelength between 470 and 490 nm) and DsRed (filter with a wavelength between 510 and 560 nm) within the cells. Subsequently, all images obtained were edited with the aid of the software AxioVision and Microsoft Office Picture Manager.

Inoculation of seeds: before installing the experiments, all the seeds were disinfected with a sodium hypochlorite solution (1%) for 1 min., rinsed with sterile distilled water and placed to dry, at room temperature, for 48 h. Following methodology described by Machado et al. (2012) seeds were evenly distributed into Petri dishes containing PDA culture medium, modified with mannitol solution (water potential adjusted to -1.4 MPa, designated by Michel and Radcliffe, 1995), and fungal colonies developed for five days; as well as each of four transformed isolates of *S. maydis* (MCL698-GFP, MY2-GFP and MCL698-DsRed, MY2-DsRed); and four transformed isolates of *S. macrospora* (CMLAPS375-GFP, CMLAPS10-GFP and CMLAPS375-DsRed, CMLAPS10-DsRed). Besides of mannitol solution addition, to the PDA medium, the antibiotic hygromycin-B was also added, at a concentration of 100 $\mu g.mL^{-1}$; and plates containing seeds were then incubated at 25±2 °C, and photoperiod of 12/12 h, during different periods (inoculum potential) [P1 (24 h); P2 (48 h); P3 (72 h); and P4 (96 h)]. For the control treatment seeds inoculated with non-transformed isolates of *S. maydis* (isolate CML698) and *S. macrospora* (isolate CMLAPS375) were used; which were also used to assess behavior of the fungus in the seeds through SEM.

Preparation of inoculated seeds for analysis by SEM: the seeds inoculated with the non-transformed isolates of *S. maydis* and *S. macrospora* were randomly chosen for observation under a scanning electron microscope. To that, seeds were transversely, longitudinally, and medially cut for the fixation in a modified Karnovsky solution (2.5% of glutaraldehyde; 2.5% formaldehyde diluted in 0.05 M sodium cacodylate buffer (pH 7.2) and CaCl2 0.001 M), at 4 °C, for 48 h. After fixation, the fragments of seeds of each of the treatments were immersed in a 30% glycerol solution, for 30 min. Subsequently, these fragments were cut in smaller pieces using liquid nitrogen, and immediately immersed in distilled water. After such process, samples were immersed in a fixative solution consisting of osmium tetroxide (1%) diluted in distilled water, for 3 h. Immediately after fixation, the samples were again washed in distilled water and then dehydrated in a series of successive dilutions of acetone [three times in pure acetone (100%); and once in solutions containing 90%, 75%, 50%, and 25% acetone]. Then the samples were transferred to a Critical Point apparatus (Balzers; CPD-030) for replacement of acetone by CO2 and finalization of drying process. The specimens so obtained were mounted on aluminum stubs, fixed with the aid of a double-sided carbon tape, and placed on an aluminum pellicle, covered with gold in a Balzers evaporator (model SCD 050), and then observed through a scanning electron microscope (Zeiss, model LEO EVO 40 PVX). The images digitally generated by this equipment, were then recorded and registered.

Results and Discussion

Genetic transformation of *S. maydis* and *S. macrospora* with the expression of GFP and DsRed fluorescent proteins was effectively performed, obtaining in the end, stable transformants, as evidenced by resistance to the antibiotic hygromycin-B and of the visualization of the fluorescent proteins, green and red.

Stable protoplasts in sufficient quantity for transformation were obtained, observing that there are several influencing factors in obtaining it, for example, the enzymatic preparation, the osmotic stabilizer, the age of mycelium, and the

microorganism to be used (Almeida et al., 2008; Ishikawa et al., 2010). Even for *S. macrospora*, which was the first attempt to produce protoplasts, the quantities were favorable, ranging between 30-45 x 10^5 protoplasts/mL, and to *S. maydis*, of 40-45 x 10^5 protoplast/mL. For many fungi this is a critical point because without obtaining intact protoplasts, the success of the transformation was questionable (Marchi et al., 2005; 2006).

With the transformation procedure, 21 transformed isolates of *S. maydis* containing the gene expressing for GFP and 20 containing the gene for DsRed, were obtained i.e., about 3-4 transformants of *S. maydis* per µg of plasmid DNA. For *S. macrospora*, 18 isolates were obtained with the GFP and 15 with the DsRed, resulting from 2-3 transformants per µg plasmid DNA. As the morphological characteristics, when comparing transformed and non-transformed isolates, these revealed few differences, as in color of the colony, being observed in older colonies (with 8-15 days) of these transformed, a darker color than normal and, sometimes, greenish tones. Concerning mycelial growth on PDA medium added with hygromycin-B, it was observed that the transformed were slower, taking 2-3 days more to fill a 90 mm Petri dish than a non-transformed isolate. On the OA culture media, added with hygromycin-B, several transformed isolates, both of *S. maydis* as of *S. macrospora* grew normally, but did not maintain the fluorescence. However, Xiao et al. (2013) report that the GFP does not interfere with normal cell functions, and therefore can be used for analysis of cellular processes. In observing the stability of mycelial growth, when the transformed fungi of both species were successively transferred for five times in PDA medium containing hygromycin-B (100 g/mL), it was verified a normal growth, and their respective fluorescence maintained. About 20% have lost this ability, which may be related to the difficulty of cellular nuclei of maintain and pass the information.

For the transformed isolates, expressing GFP (Figure 1) and DsRed of *S. maydis* and *S. macrospora*, green and red fluorescence were observed in the cytoplasm of hyphae and conidia; however, the pycnidia retained their natural color, even if their conidia were positive. However, there has been a variation in fluorescence intensity both for the green as the red. The lowest levels of fluorescence intensity were found in the transformed isolates expressing DsRed.

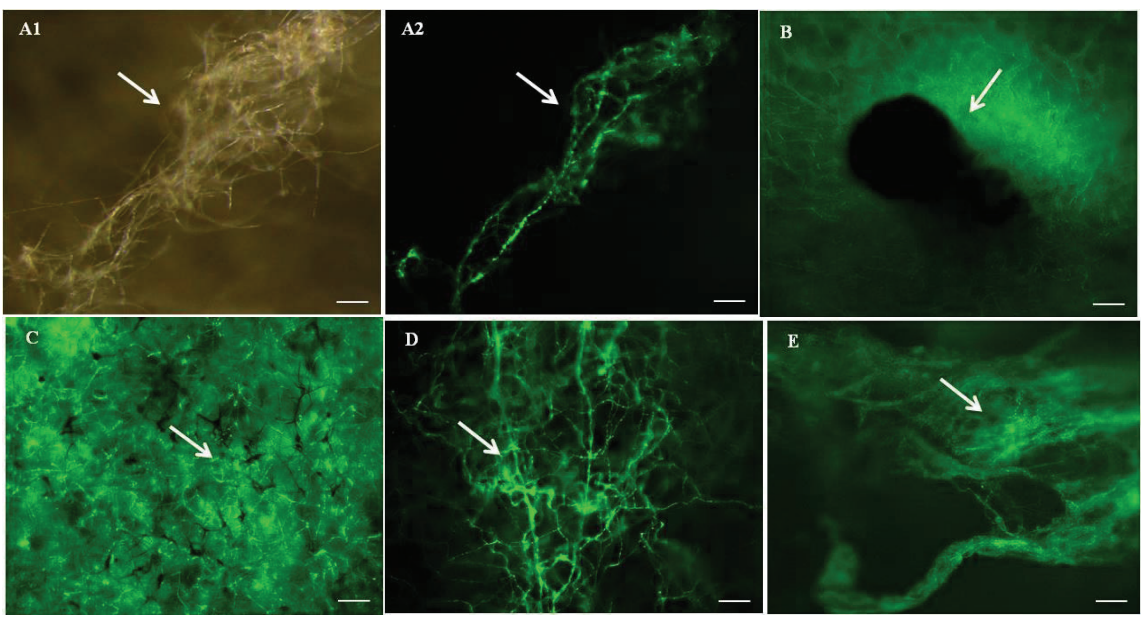

Figure 1. Stereo micrographs of fluorescence of the fungi marked with green fluorescent protein, GFP. To *Stenocarpella maydis*: A1 (white light), A2 and C (hyphae of the isolate CML698-GFP) and B (pycnidia and hyphae of the isolate MY2-GFP). To *Stenocarpella macrospora*: D (hyphae of the isolate CMLAPS10-GFP), E (hyphae of CMLAPS375-GFP). Bar = 200 µm.

In the seeds inoculated with the transformed isolates, expressing GFP, direct correlations between the fluorescence intensity of the colonies with the inoculum potential (time of exposure of the seeds to the transformed fungus) (Figure 2) were observed. In the seeds inoculated with P4 (96h) it was possible to observe an abundant and compact mycelial growth, evidenced by the strong fluorescence emitted by the transformed fungus; which allowed to visualize and

to monitor the development of the infection process. In a study performed with maize seeds infected by the fungus *Aspergillus flavus*, Du et al. (1999) have correlated the intensity of the fungus mycelial fluorescence with the presence and the intensity of seed colonization by this transformed organism. They also emphasized the importance of using this tool on detecting this fungus in kernels meant to food and for selecting maize genotypes resistant to accumulation of aflatoxins, which are toxic substances produced by this fungus. Therefore, this study may be used as basis for further investigations correlating colonization of seeds for transformed *Stenocarpella* species with the presence of harmful toxins produced, such as diplodiatoxin produced by *S. maydis*, and toxin diplodiol produced by *S. macrospora* (Petatán-Sagahón et al., 2011) for which there is little information in the literature.

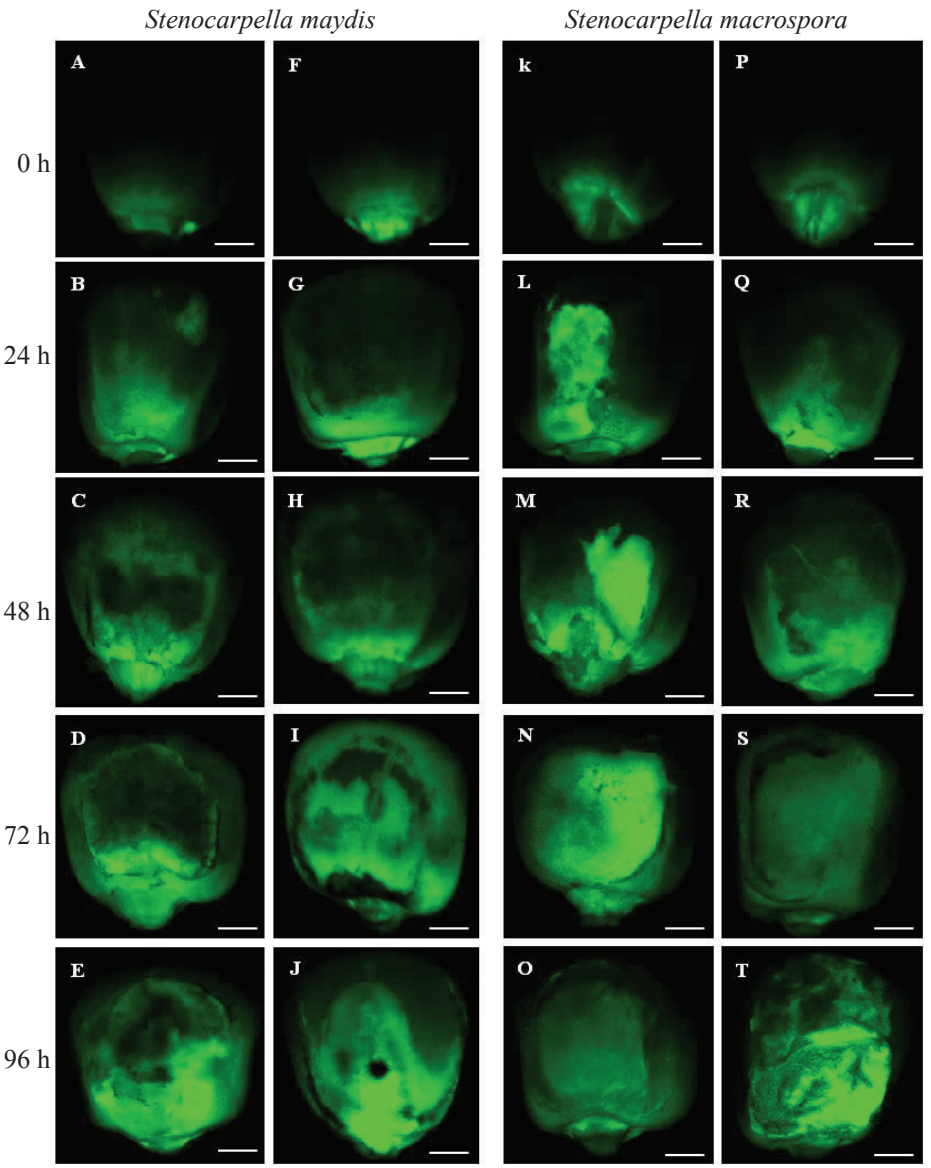

Figure 2. Stereo micrographs of the maize seeds inoculated with the fungi *Stenocarpella maydis* and *Stenocarpella macrospora* marked with GFP with different inoculum potential [P1 (24 h), P2 (48 h), P3 (72 h), and P4 (96 h)]. Seeds with transformed *Stenocarpella maydis*, isolated CML698-GFP (B, C, D and E) and MY2-GFP (G, H, I and J). Seeds with transformed *Stenocarpella macrospora*, isolated CMLAPS375-GFP (L, M, N and O) and CMLAPS10-GFP (Q, R, S and T). Seeds of controls treatment, or without inoculation: A, F, K and P. Bar = 2 mm.

There were no major visual differences between the seeds inoculated with transformed isolates of *S. maydis* or *S. macrospora*; however, it was possible to observe stronger fluorescence intensity on the seeds inoculated with *S. maydis*. These results may be due to the fact that this species of the fungus grows easily in the maize seeds; whereas the species *S. macrospora* consistently associated with growth on maize leaves (Casa et al., 2006).

Seeds inoculated with transformed isolates of *S. maydis* and *S. macrospora* expressing the DsRed protein did not differ from seeds inoculated with isolates expressing the GFP (Figure 3). However, it was observed that seeds inoculated with the isolates expressing DsRed with higher inoculum potential (P3 and P4) had more fluorescence; thereby allowing to conclude that in this inoculum potential occurred a deeper infection, which have reached even the embryo and the endosperm and that, when observed the red fluorescence was much more evident on inoculated seeds, especially when compared to non-inoculated seeds (control) (Figure 4).

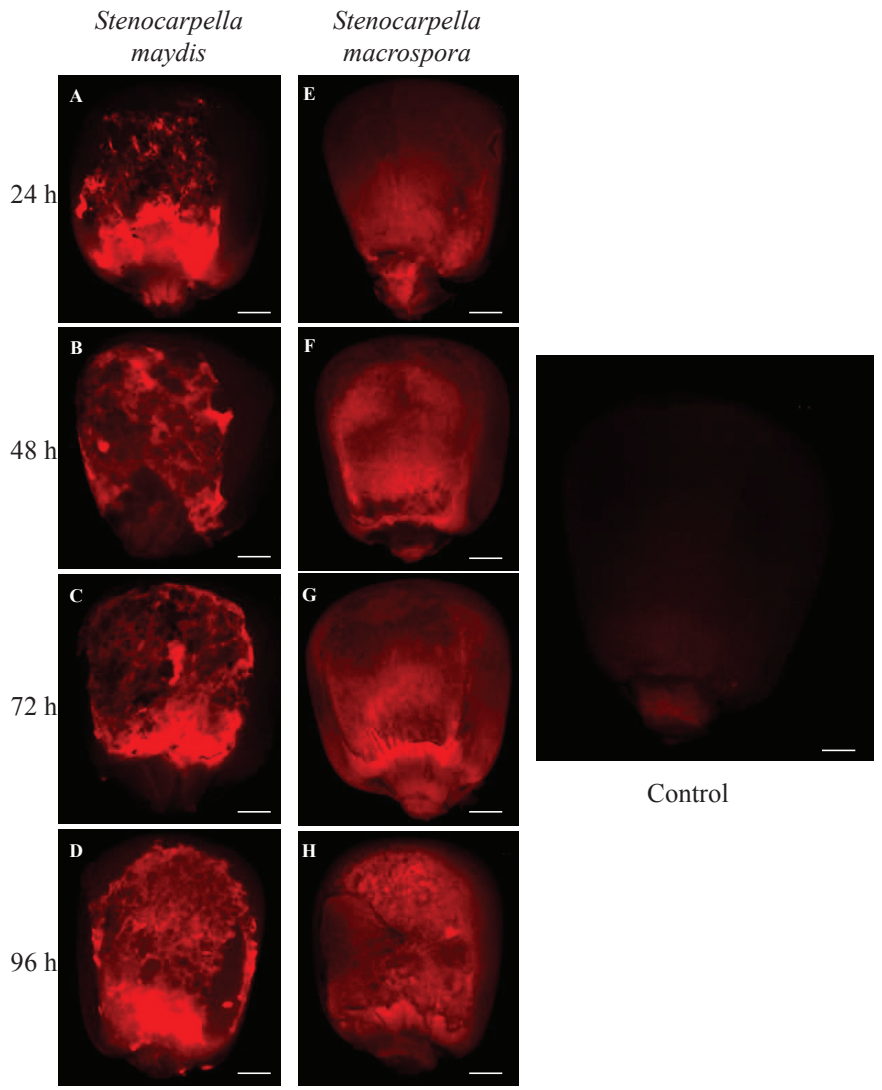

Figure 3. Stereo micrographs of the maize seeds inoculated with the fungi *Stenocarpella maydis* and *Stenocarpella macrospora* marked with DsRed with different inoculum potential [P1 (24 h), P2 (48 h), P3 (72 h), and P4 (96 h)]. Seeds with transformed *Stenocarpella maydis*, isolated CML698-DsRed (A, B, C and D). Seeds with transformed *Stenocarpella macrospora*, isolated CMLAPS375-DsRed (E, F, G and H). Seed of control treatment, or without inoculation (Control). Bar = 2 mm.

Besides observing that the transformed isolates, both of *S. maydis* as of *S. macrospora*, expressing the GFP and DsRed proteins are able to show infection and colonization, seeds were also assessed by SEM; and through this tool it was

possible to observe in details the seed colonization process by these two pathogens.

Through SEM it was also possible to assess changes in the characteristics of the main physical structures of maize seeds [endosperm, germ, pericarp, and tip (Paes, 2006)] and it was found that these changes in cellular structures were directly proportional to the increase of inoculum potential in the seeds inoculated with *S. maydis* and *S. macrospora*.

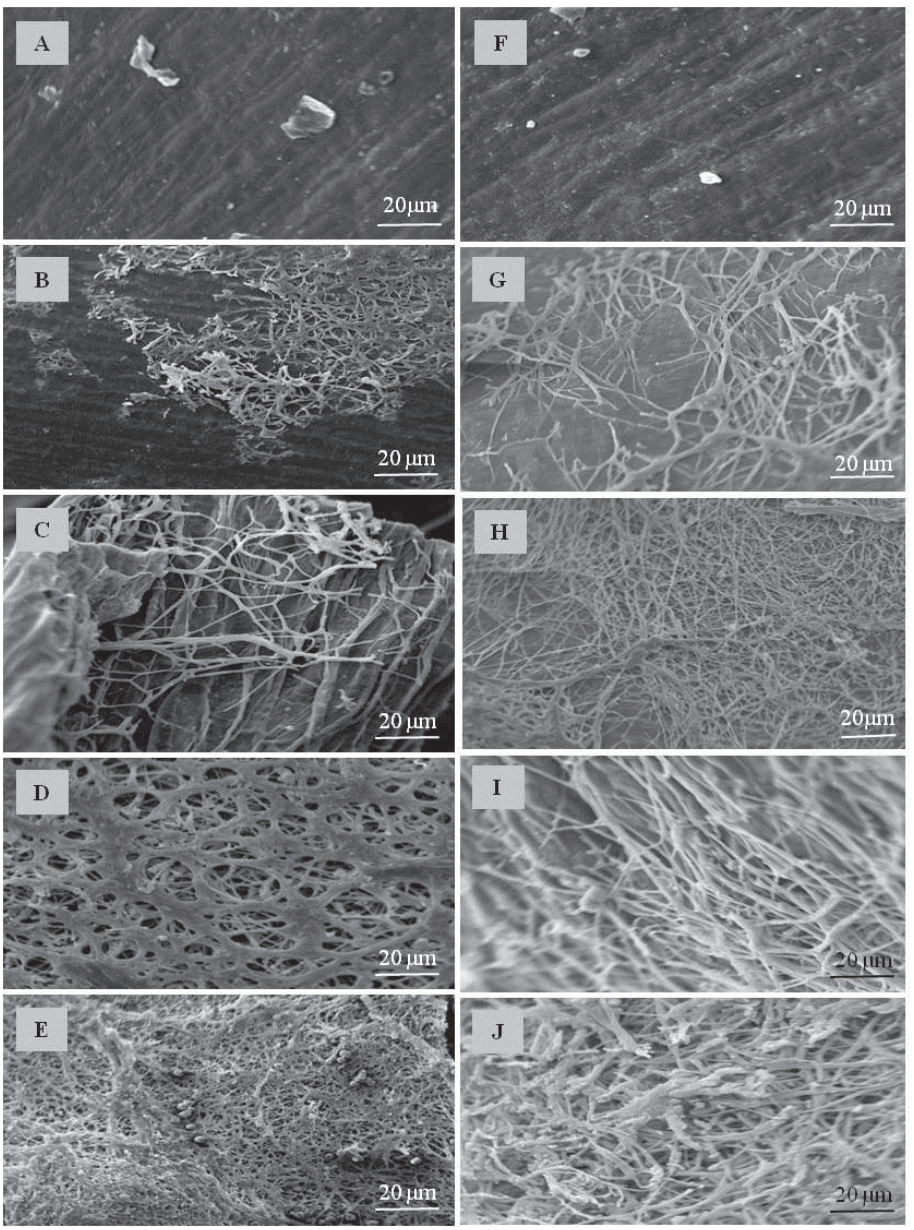

Figure 4. Electron micrographs of the epidermis of maize seeds inoculated with *Stenocarpella maydis* (B, C, D, E) and *Stenocarpella macrospora* (G, H, I, J). A and F= Control or non-inoculated seed; G and B = inoculum potential P1 (24 h); C and H= P2 (48 h); D and I= P3 (72 h); E and J=P4 (96 h).

In the pericarp, where are the epidermis (first layer), followed by the mesocarp, cross cells, tubular cells and testa (last layer of pericarp), in all inoculation periods, it was observed the presence of the species of *Stenocarpella* (Figure 4). In P1, the hyphae of the isolates were more superficial and have caused deformations in the epidermis; in P2, hyphae were found behind the testa, which demonstrates the ability rapidity with which of the colonization by the two fungi in crossing the pericarp of the seeds. However, in the higher inoculum potentials (P3 and P4) it was observed the colonization of

almost all the pericarp, with large quantities of mycelium, and this was becoming more compact and well adhered to the layers, causing drastic reduction in the volume of the protection cells, with injuries and deformation of the outer parts of the seeds. The loss of integrity of the pericarp can affect the electrical conductivity of the exudates released by the maize seeds. The same can also be observed in seeds that are submitted to a drastic drying process (José et al., 2005). No major differences were observed between the colonization of *S. maydis* and *S. macrospora*, but with *S. macrospora* it was verified that mycelium was more aerial on the seed surface or less adhered to the seeds and slightly thicker.

The combined effect of those pathogens in the inner parts of the maize seeds is of great importance because the endosperm represents about 83% of the dry weight of the maize kernels (Figures 5 and 6). The endosperm is constituted especially of starch (88%), and arranged in the form of *granules* and of storage proteins (8%) of prolamin type, called zeins, these proteins form the *protein bodies* that compose the matrix which surrounds the starch granules within the endosperm cells (Paes, 2006), i.e., the major part of the maize seed. Moreover, the species of *Stenocarpella* may take advantage of the inner of the maize seeds, based on the distribution of the starch granules and of matrix proteins that compose the endosperm classified as: farinaceous and vitreous. In the first, the starch granules are rounded and dispersed with no protein matrix surrounding these structures, which results in vacant spaces during the drying process of the seed, starting from the spaces where it previously was occupied by the water, during the development of the seed (Paes, 2006). These spaces may be occupied entirely by masses of hyphae, as observed in P3 and P4, for both species, waiting for favorable conditions to develop, besides being responsible for the damages caused to the seeds. However, in the vitreous endosperm, the protein matrix is dense, with structured protein bodies and surrounding the polygonal format starch granules, and with no spaces formation among these structures. Under such conditions it was observed hyphae very adhered in to deformed granules and developing among the protein bodies.

S. maydis and *S. macrospora* were able to start the infection process and to develop themselves in the maize seeds with any inoculum potential; as demonstrated in this study. In all inoculum potentials dormant structures of the fungus were found, and from there those structures may develop in the presence of water, nutrients and favorable temperature, culminating to disease development leading often to death the seeds/seedlings, as expected at higher inoculum potentials.

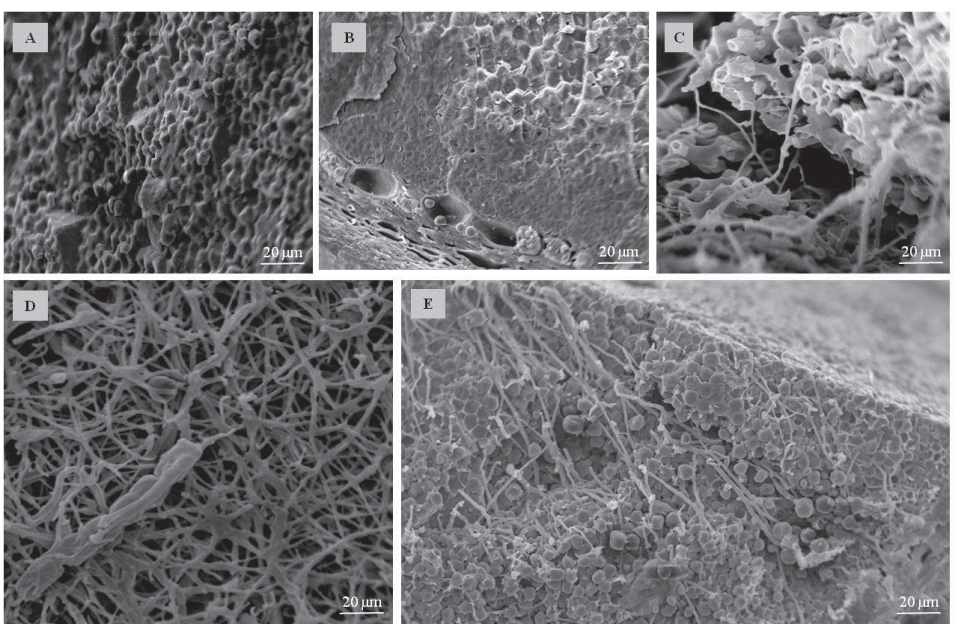

Figure 5. Electron micrographs of the inner parts of a maize seed inoculated with *Stenocarpella maydis* during different periods. A= control (non-inoculated seed); B= P1 (24 h); C= P2 (48 h); D = P3 (72 h); E = P4 (96 h).

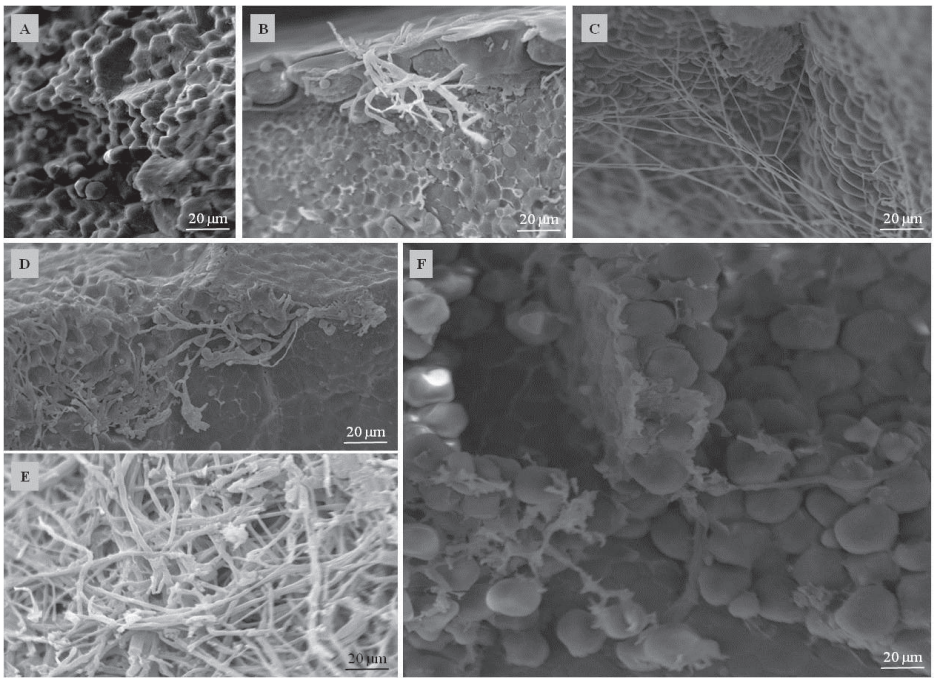

Figure 6. Electron micrographs of the inner parts of a maize seed inoculated with *Stenocarpella macrospora* during different periods. A= control (non-inoculated seed); B e C = P1 (24 h); D = P2 (48 h); E = P3 (72 h); F = P4 (96 h).

Conclusions

The protocol used for production of protoplasts and incorporation of genes that express GFP and DsRed in *S. maydis* and S. *macrospora* is efficient and satisfactory for the transformation of those species and for its use in seed pathology studies.

Seeds inoculated with isolates of genetically transformed *Stenocarpella* species elucidated, by means of the fluorescence intensities, the varying levels of infection and colonization found in the different inoculum potentials.

Analyzes performed by SEM on maize seeds inoculated with *S. maydis* and *S. macrospora* were effective for demonstrating the damage caused to the tissues of the seeds by the infection and colonization, being the damages more severe when observed in the higher inoculum potentials.

Acknowledgments

To CNPq, CAPES and FAPEMIG for financial support for conducting this study; to Dr. Theo van der Lee (Plant Research International, The Netherlands) who provided plasmids used for fungal transformations; to company Riber Seeds (Patos de Minas/MG); to Plant Virology laboratory and Electron Microscopy and Ultrastructural Analysis laboratory of Department of Plant Pathology, Federal University of Lavras (Lavras/MG).

References

ALMEIDA, A. P. M. M.; DIAS, E. S.; PEREIRA, R. T. G.; TOLEDO, R. C. C.; PFENNING, L. H. Obtenção de protoplastos do fungo filamentoso *Aspergillus ochraceus*. *Ciência Rural*, v.38, n.5, p.1460-1462, 2008. http://www.scielo.br/pdf/cr/v38n5/a43v38n5.pdf

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. *Regras para análise de sementes*. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Brasília: MAPA/ACS, 2009a. 395p. http://www.agricultura.gov.br/arq_editor/file/2946_regras_analise__sementes.pdf

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. *Manual de Análise Sanitária de Sementes*. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Brasília: MAPA/ACS, 2009b. 200p. http://www.agricultura.gov.br/arq_editor/file/12261_sementes_-web.pdf

CASA, R. T.; REIS, E. M.; ZAMBOLIM, L. Doenças do milho causadas por fungos do gênero *Stenocarpella*. *Fitopatologia Brasileira*, v.31, n.5, p.427-439, 2006. http://www.scielo.br/pdf/fb/v31n5/01.pdf

CRISTEA, I. M.; WILLIAMS, R.; CHAIT, B. T.; ROUT, M. P. Fluorescent Proteins as Proteomic Probes. *Molecular & Cellular Proteomics*, v.4, n.12, p.1933-1941, 2005. http://www.mcponline.org/content/4/12/1933.full.pdf

DU, W.; HUANG, Z.; FLAHERTY, J. E.; WELLS, K.; PAYNE, G. A. Green fluorescent protein as a reporter to monitor gene expression and food colonization by *Aspergillus flavus*. *Applied and Environmental Microbiology*, v.65, n.3, p.834-836, 1999. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC91103/pdf/am000834.pdf

ISHIKAWA, F. H.; BARCELOS, Q. L.; SOUZA, E. A.; DIAS, E. S. Factors affecting the production and regeneration of protoplasts from *Colletotrichum lindemuthianum*. *Ciência e Agrotecnologia*, v.34, n.1, p.74-79, 2010. http://www.scielo.br/pdf/cagro/v34n1/09.pdf

JOSÉ, S.C.B.R.; VON PINHO, E. V. R.; VON PINHO, R. G.; RAMALHO, M. A. P.; FILHO, J. L. S. Características físicas do pericarpo de sementes de milho associadas com a tolerância à alta temperatura de secagem. *Revista Brasileira de Sementes*, v.27, n.1, p.125-131, 2005. http://www.scielo.br/pdf/rbs/v27n1/25189.pdf

MACHADO, J. C.; BARROCAS, E. N.; COSTA, M. L. N.; GUIMARÃES, R. M.; MACHADO, C. F. Uso da técnica de restrição hídrica ou condicionamento osmótico em patologia de sementes. *Revisão Anual de Patologia de Plantas*, v.20, p.1-24, 2012.

MAIER, F. J.; MALZ, S.; LOSH, A. P.; LACOUR, T.; SCHAFER, W. Development of a highly efficient gene targeting system for *Fusarium graminearum* using the disruption of a polyketide synthase gene as a visible marker. *FEMS Yeast Research*, v.5, n.6/7, p.653-662, 2005..http://dx.doi.org/10.1016/j.femsyr.2004.12.008

MAOR, R.; PUYESKY, B. A.; SHARON, A. Use of green fluorescent protein (GFP) for studying development and fungal-plant interaction in *Cochliobulus heterostrophus*. *Mycology Research*, v.102, n.4, p.491-496, 1998. http://www.sciencedirect.com/science/article/pii/ S0953756208609053

MARCH, J.C.; RAO, G.; BENTLEY, W.E. Biotechnological applications of green fluorescent protein. *Applied Microbiology and Biotechnology,* v.62, p.303–315, 2003. http://download.springer.com/static/pdf/775/art%253A10.1007%252Fs00253-003-1339y.pdf?auth66=1382536505_d1804f14bb2deaf1300b5672930dd86b&ext=.pdf

MARCHI, C. E.; BORGES, M. F.; BROMMONSCHEKEL, S. H. Obtenção de protoplastos de *Botryosphaeria* sp. *Ciências Biológicas e da Saúde*, v.12, n.4, p.23-32, 2006. http://www.revistas2.uepg.br/index.php/biologica/article/view/441/442

MARCHI, C. E.; BROMMONSCHENKEL, S. H.; QUEIROZ, M. V.; MIZUBUTI, E. S. G. Isolation and regeneration of *Magnaporthe grisea* protoplasts. *Summa Phytopathologica*, v.32, n.3, p.232-238, 2005. http://www.scielo.br/pdf/sp/v32n3/a04v32n3.pdf

MICHEL, B. E.; RADCLIFFE, D. A computer program relating solute potential to solution composition for five solutes. *Agronomy Journal*, v.87, n.1, p.131-136, 1995.

PAES, M.C.D. *Aspectos físicos, químicos e tecnológicos do grão de milho.* EMBRAPA/CNPMS, 2006. 14p. (Circular técnica, 75). http://www.cnpms.embrapa.br/publicacoes/publica/2006/circular/Circ_75.pdf

PETATÁN-SAGAHÓN, I.; ANDUCHO-REYES, M. A.; SILVA-ROJAS, H. V.; ARANA-CUENCA, A.; TELLEZ-JURADO, A.; CÁRDENAS-ÁLVAREZ, I. O.; MERCADO-FLORES, Y. Isolation of bacteria with antifungal activity against the phytopathogenic fungi *Stenocarpella maydis* and *Stenocarpella macrospora*. *International Journal of Molecular Sciences*, v.12, p.5522-5537, 2011. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3189730/

RIBEIRO, N.A.; CASA, R. T.; BOGO, A.; SANGOI, L.; MOREIRA, E. N.; WILLE, L. A. Incidência de podridões do colmo, grãos ardidos e produtividade de grãos de genótipos de milho em diferentes sistemas de manejo. *Ciência Rural*, v.35, n.5, p.1003-1009, 2005. http://www.scielo.br/pdf/cr/v35n5/a04v35n5.pdf

SAMBROOK, J.; RUSSELL, D.W. *Molecular cloning*: a laboratory manual, 3.ed. Cold Spring Harbor: Laboratory Press. 2001. 2344p.

SILVA, A. P.; BOLTON, M. D.; NELSON, B. D. Transformation of *Sclerotinia sclerotiorum* with the green fluorescent protein gene and fluorescence of hyphae in four inoculated hosts. *Plant Pathology*, v.58, n.3, p.487-496, 2009. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-3059.2009.02022.x/pdf

XIAO, R. F.; ZHU, Y. J.; LI, Y. D.; LIU, B. Studies on vascular infection of *Fusarium oxysporum* f. sp. *cubense* race 4 in banana by field survey and green fluorescent protein reporter. *ESci Journal Plant Pathology*, v.2, n.1, p.44-51, 2013. http://escijournals.net/EJPP/rt/captureCite/64/0