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INFECTION OF MAIZE SILKS BY A NATIVE FUSARIUM (*Fusarium graminearum)* ISOLATE IN ARGENTINA

INFECCIÓN EN ESTILOS DE MAÍZ POR UN AISLAMIENTO NATIVO DE FUSARIUM (*Fusarium graminearum*) EN ARGENTINA

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ABSTRACT

Some species of the genus *Fusarium* cause different diseases in corn, such as stalk rot and ear rot, which affect its yield and quality. *F. graminearum* Schwabe (Teleomorph *Gibberella zeae*) is one of the prevalent species of *Fusarium* producing these diseases in the Argentinean core corn area. *Fusarium* spp. can enter the ear through the silk channel or through wounds caused by insects or birds. The objectives of this work were: 1) to evaluate the ability of a native *F. graminearum* isolate to infect maize flowers; and 2) to determine the most effective time for fungal infection event. To do this, corn ears were inoculated by spraying at 3 different times: (a) prior to fecundation, (b) on fecundation, and (c) on senescent silks. One portion of silk was stained to determine the presence of the fungus and fungal infection with an optical microscope, and the other portion was placed in acidified potato glucose agar (APGA) to verify the presence of *F. graminearum.* It was concluded that silks are receptive along all their exposed length, and penetration of *F. graminearum* takes place before and during fecundation and at silk senescence, with more infection level at fecundation and on senescent silks. *F. graminearum* presence in senescent silk tissues shows the necrotrophic ability of this pathogen and a high plasticity of adaptation to environmental conditions which are not optimal to cause infections.

Key words: *F. graminearum, F. verticillioides,* silk, physiological quality.

RESUMEN

Algunas especies del género Fusarium causan diferentes enfermedades en el maíz, como la pudrición del tallo y la pudrición de la mazorca, que afectan a su rendimiento y calidad. *F. graminearum* Schwabe (Teleomorfo *Gibberella zeae)* es una de las especies predominantes de *Fusarium* que producen estas enfermedades en la zona núcleo de maíz argentino. *Fusarium* spp. puede entrar a través del canal del estigma o heridas causadas por insectos o aves. Los objetivos de este trabajo fueron: 1) evaluar la capacidad de un aislamiento de *F. graminearum* nativo para infectar las flores de maíz; y 2) determinar el momento más efectivo para la infección por hongos. Para ello, las mazorcas de maíz se inocularon por pulverización en tres momentos: (a) antes de la fecundación, (b) en la fecundación, y (c) en estigmas senescentes. Una porción del estigma se tiñó para determinar con microscopio óptico la presencia del hongo y la infección fúngica, y la otra porción se colocó en agar papa glucosado acidulado (APGA) para verificar la presencia de *F. graminearum.* Se concluyó que los estigmas son

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receptivos a lo largo de toda su longitud expuesta, y la penetración de *F. graminearum* se lleva a cabo antes y durante la fecundación y en estigmas senescentes, con más nivel de infección en la fecundación y en estigmas senescentes. *F. graminearum* presente en los tejidos de estigmas senescentes muestra la capacidad necrotrófico de este patógeno y una alta plasticidad de adaptación a las condiciones ambientales que no son óptimas para causar infecciones.

Palabras clave: *F. graminearum, F. verticillioides*, estigmas; calidad fisiológica.

INTRODUCTION

Maize (*Zea mays* L.) crop is affected by numerous biotic diseases caused by fungi, bacteria and virus (Goswami and Kistler, 2004). *Fusarium, Diplodia and Penicillium* are some of the fungal pathogens that cause rots on root, stalk and mainly on the ear, where the grains or caryopsis are covered by the mold affecting their commercial and physiological quality (Incremona et al., 2008; Govender et al., 2008).

The genus *Fusarium* is particularly important because it includes several species capable of producing ear and basal rotting in maize; among them: *F. graminearum* Schwabe, section: discolor *F. roseum* Link emend. Snyder and Hansen teleomorph: *Gibberella zeae*) (Beyer and Rabag, 2005) (*F. verticillioides* (Saccardo) (= *F.moniliforme* Sheldon, section: Liseola; teleomorph: *Gibberella fujikuroi*) (*Sawada G. moniliformis* Wineland) (Covarelli et al., 2012) y *F. oxysporum* Schlectend: Fr. Section: Elegans (Dejardins, 2003; Leslie et al., 2006). *F. graminearum*, one of the most significant causal agents of the maize ear rot (White, 1999), produces symptoms at the tip and occasionally at the lower third of the ear.

The pathogen uses different ways to start the infection: through flower silks (Lewandowski et al., 2006) or by wounds made by insects or birds. Even though the studies on the time and way that *F. graminearum* natural infection occurs in maize flowers are not abundant, it seems the pathogen is able to enter to style channel when the silks have just elongated and pollinated. This phenologic event can occur between six and seven days after the silk emergence, which is produced from the lower third to the tip of the ear. After penetration, *F. graminearum* mycelium starts to grow in silks and spreads along the silks to the caryopsis serving as a substrate for fungal growth (Schaafsma et al., 2005).

On the other hand, many studies regarding the infection process of *F. graminearum* caused by injection of conidia in the ears show a high effectiveness in the spread of ear rot (Chungu et al., 1996). The severity of the disease and the concentration of mycotoxins such as deoxynivalenol (DON) (Del Ponte et al., 2007) are more evident in susceptible maize genotypes and high amounts of inoculum of *F. graminearum* conidia (Blandino et al., 2012). The highest susceptibility of corn silk to *F. graminearum* infection was observed after silk emergence and tended to decrease with age, occurring between the fifth and seventh day after pollination (Reid et al., 1999). This behavior could be explained by several facts, such as the turgency of young silks, the reaction of genotypes of maize and fungi and environmental interactions. The same authors reported also that *F. verticillioides* can suppress the growth of *F. graminearum* in maize ears due to a negative association between both *Fusarium* species. The maize pollen would stimulates the macronidia germination, germ tube growth and colonization of silks during the *F. graminearum* infection (Lewandowski et al., 2006).

On the other hand, insects and birds use caryopsis as food and the damage caused predisposes the fungal infection and ear rot. Infection is very aggressive and produces high severity levels (Clements et al., 2003)

The ear rots caused by natural infection or infection by artificial spray of *F. graminearum* spores are less aggressive and severe than those caused by wounds (Clements et al., 2003; Presello et al., 2006). This fact would suggest that silks can be the initial barriers of resistance preventing penetration of different fungal strains and environmental conditions.

The objectives of this work were: 1) to evaluate the ability of a native *F. graminearum* isolate to infect maize flowers; and 2) to determine the most effective time for fungal infection event.

MATERIALS AND METHODS

Two trials to determine the ability to infect maize silks and to assess the infection time of a virulent *F. graminearum* native isolate were conducted at the Experimental Field of Agronomy School, University of Rosario, Zavalla (Santa Fe Province, Argentina 33°01′ S.; 66°53′O.).

Experiment 1st year. Plant material. Two commercial maize hybrids (PO1024 and PO1170) with different compression of the husks and behaviors against ear pathogens were used. Original seeds of both genotypes, without fungicide treatment, were sown according to a split plot design with three replications. Each replication (plot) consisted of four rows spaced at 0.70 m. and 3 m long.

There were three rows for each hybrid and plot with five plants spaced at 0.20 m each row, resulting in 15 plants per hybrid and replication (Incremona et al., 2008).

Inoculum production and inoculation. *F. graminearum* isolate (CE170) used in this study had been previously characterized by morphological and molecular markers and pathogenical capacity (Pioli et al., 2005) and it was kept in the Mycological Reference Center (CEREMIC) of Biochemistry and Pharmacy School of National University of Rosario. *F. graminearum* was grown in plates with Acidified Potato Glucose Agar 2% (APGA) (Fig. 1A) and SNA (synthetic nutrient agar) medium to promote its sporulation (Fig. 1B) (Leslie et al., 2006). A suspension with 2×10^5 conidia mL^{-1} of sterile water was obtained for inoculating the ears at the appropriate moments. Ten plants per hybrid and replication were inoculated with *F. graminearum* and 2 control plants were sprayed with sterile water. Inoculated silks of each ear were sprayed with 2 mL of spore suspension and the same volume of sterile water was sprinkled over the control plants. Ears were covered with polyethylene bags to favor 100% humidity for 72 h after inoculation.

Evaluation of ability and moment to infect silks by *F. graminearum*. The process was assessed through the histological changes of plant tissues simultaneously to the progress of the infection process caused by *F. graminearum* at three different times in both PO1024 and PO1170 hybrids.

a. Inoculations previous to plant pollination. Maize female flowers were covered with a paper bag before silk emergence to avoid pollination, and when the ears were in the early stage of 50% of silk emergence (R1) were uncovered (Fig. 2A). Emerged silks were sprayed with the fungal inoculum and covered with a plastic bag for 48 hours to maintain the humidity and stimulate the entry of the pathogen. Inoculated silk were collected on the 1st, 4th and 6th day after inoculation (dai) for histopathological studies. The 5 cm upwards from head tip of the inflorescence were cut indicating the pistil basal extreme by a bevel cut (Fig. 2B) and were conserved in sterile water or alcohol

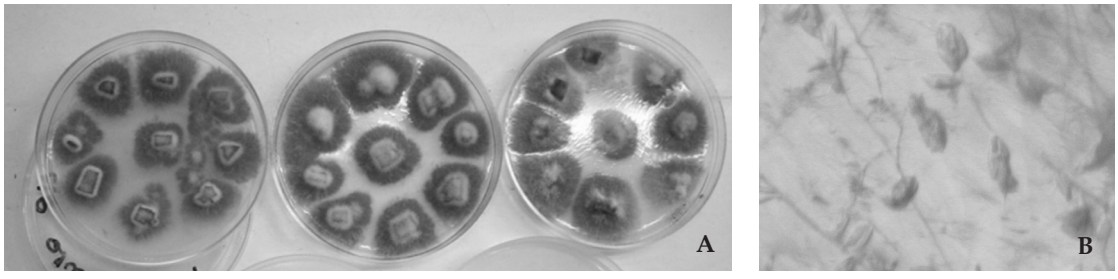

Fig. 1. A: *F. graminearum* colonies obtained on APGA medium (40x), and B: *F. graminearum* sporodoquia and macro conidia obtained on SNA medium.

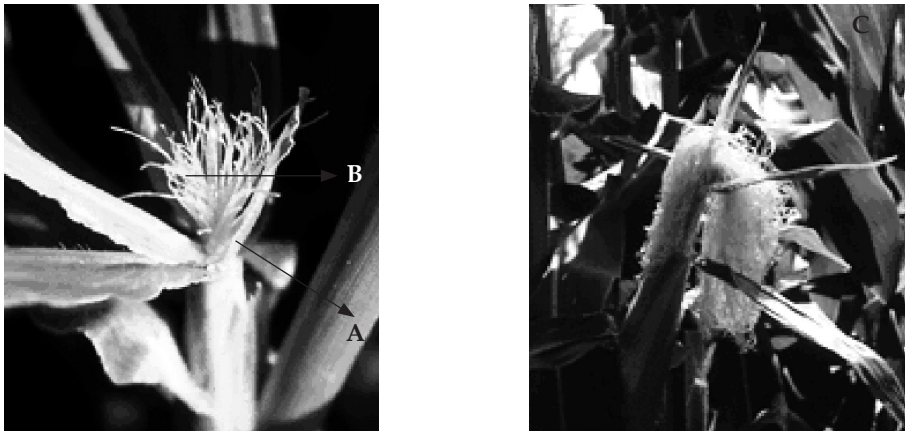

Fig. 2. Female inflorescence of maize flowers at silk emergence. A: Silk emergences from the head tip of the ear; B: The arrow shows the cut of the silks for inoculation; C: Silks are observed a week after emergence.

70°. Then, the rest of the silks left in the ear were artificially pollinated and covered again with paper bag to avoid contamination of caryopsis with other pathogens.

b. Inoculations during plant pollination. After the ears began to show 50% of emerged silk, they were pollinated and further inoculated. Then, they were covered with a plastic bag for 48 hours to stimulate the infection. The samples of silks were taken at 4th and 5th day after inoculation and processed of same way. Ears were covered again with paper bag to avoid contamination of caryopsis with other pathogens.

c. Plants with senescent silk tissues. Senescent silks were inoculated on the 10th day after ear pollination (Fig. 3) and covered for 48 hours. Silks were harvested at 3th day and processed of the same way. Ears were covered with paper bag to avoid contamination of caryopsis with other pathogens.

Histopathological techniques. A sample of 13 inoculated silks per ear was selected (130 silks per replication and hybrid; total: 390 silks per hybrid). A sub-sample with 10 inoculated silks per ear (100 silks per replication and hybrid; total 300 per hybrid) was placed on Petri dishes with APGA in order to isolate *F. graminearum* colonies. These harvested silks were washed in 1% sodium hypochlorite for 30 seconds and were dried on filter paper. Three or four silks were placed in each Petri dish with APGA medium according to their length. Incubation was at 25°C with a 12 h photoperiod during 7 days. Colonies were identified by their macro and micromorphology.

The other sub-sample with 3 inoculated silks per ear (30 silks per replication and hybrid; total 90 per hybrid) was conserved in alcohol 70° and lactophenol solution to observe later hypha invasion. Silk sample was treated with a 5% Na OH solution and 2 g of trypan blue at approximately 20°C for 22 to 24 h; then, they were washed with hot water. Silks were clarified in fresh lactophenol solution (water-free) while they were boiled for approximately 30 seconds (Incremona et al., 2008). Later, silks were examined in fresh glycerol for the histological studies; an arbitrary rating scale, expressed in μm, was used for measurements of silk tissues.

Experiment 2nd. year. Another trial with the same hybrids (PO1024 and PO1170) and similar crop management and experimental design was performed in the following year. Inoculations were also made in the same three moments: previous, during, and after pollination onto senescent silk tissues.

Data were analyzed by a factorial ANOVA, transformed to arcsine $\sqrt{x}$ and expressed in percentages. Duncan's and F tests at 5% of probability were used to compare means of treatments. The analysis was conducted with the INFOSTAT (2006), software program.

RESULTS

The histological structure of a floral silk of maize (Fig. 4) shows the superficial pilosity and internal vascular bundles. This study showed that the *F. graminearum* hyphae can penetrate the silk through both the forked apex and the hairs, demonstrating that silks are receptive on all their length (40x) (Fig. 4).

Moments of inoculation and effective infection time: *F. graminearum* incidence in silks under a lactophenol treatment. *F. graminearun* hyphae were stained according to their growth, showing blue or light brown color for active and senescent hyphae, respectively. Floral silks obtained from ears inoculated in three different moments (before and during pollination, and senes-

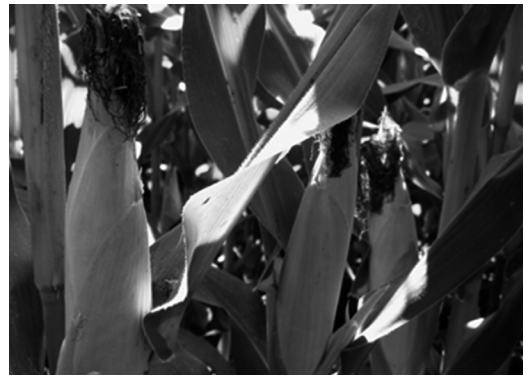

Fig. 3. Female inflorescence of maize flowers showing senescent silks.

cent tissues) showed active and senescent hyphae colonizing the basal tip (100x) (Fig. 5 (A/D). Table 1 shows *F. graminearum* incidence (%) on infected silks in the three inoculation moments. There was no significant interaction between the hybrids of maize and the moments of inoculation (p = 0.8953). Although, there were significant differences among the inoculation moments, the ears inoculated before pollination and the senescent floral silk showed the greatest infection values.

Silks *F. graminearum* incidence evaluated in APGA. Silks collected from ears inoculated at three different moments of the pollination process, for both hybrids maize (PO1024 and PO1170), developed *F. graminearum* colonies in APGA (Fig. 6). These results reveled that infections of *F. graminearum* were effective in the three inoculation moments. Fig.6 A/B shows a high incidence of infected silks with *F. graminearum* in ears inoculated before pollination. However, *F.*

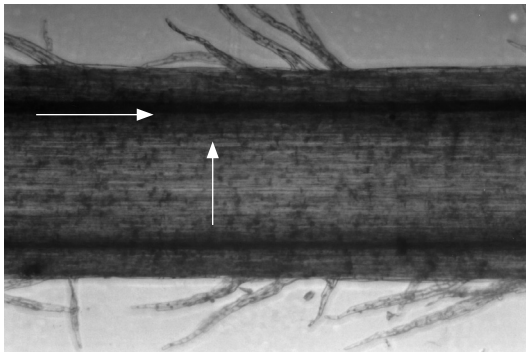

Fig. 4. Silk morphology of maize flower, presence of vascular bundles that go over the whole silk and pilosity on its surface.

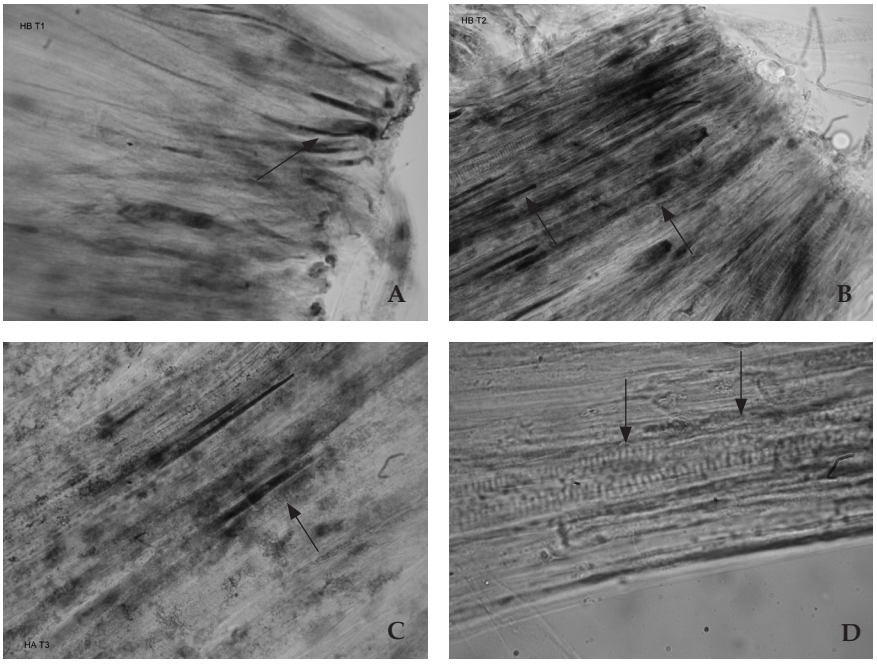

Fig. 5. *F. graminearum* hyphae in silks of maize flowers inoculated before and during pollination and in senescent silk tissues, under a lactophenol treatment. A: *F. graminearum* hyphae in a plant silk were inoculated before pollination (100x); B: *F. graminearum* hyphae in plant silks were inoculated during pollination (100x); C-D: observed hyphae in senescent silk tissues (40x); A-C black arrows point to active hyphae (in blue), A and D red arrows points to senescent hyphae (in brown).

Table 1. Incidence (%) of *F. graminearum* in silks of maize clarified in lactophenol solution and infected before and during pollination and in senescent silk tissues (2005-2006).

Hybrids	Time of inoculation with *F. graminearum*			Means
	Before fecundation	During fecundation	Senescent stigma tissues	
PO1024	75	55	72	67 a
PO1170	69	54	64	62 a
Media	72 A*	55 B	68 A	

* Means followed by the same lower case letter in the column do not differ, and means followed by the same capital letter in the row do not differ at the 5% level (F test).

graminearum was also isolated from ears inoculated during pollination (Fig. 6 C), and associated to senescent silk tissues (Fig. 6 D). Floral silks used as controls presented only bacteria colonies (Fig. 6 E). On the other hand, *Alternaria* spp., *Epicoccum* spp., *Aspergillus niger*, *A.flavus* and *Epicoccum* spp. were isolated as saprophytic and secondary (less important than *F. graminearum*) fungi from silk tissues during and after pollination.

Incidence (%) of infected silks by *F. graminearum* obtained from ears inoculated before and during pollination and in senescent silk tissues is shown in Table 2. There was a significant interaction between hybrids and inoculation moments ($p < 0.0001$). Plant behavior was different between both maize hybrids and between moments of inoculation. PO1024 presented higher susceptibility to infection than PO1170 for the three moments of inoculation. Ears inoculated before pollination and silks infected during pollination showed, respectively, the highest (30) and lowest (5) infection values in the hybrid PO1024. Meanwhile, ears inoculated with senescent silk showed the highest percentage of infection by *F. graminearum* in the hybrid PO1170, in relation to other inoculation moments. Disease was present from the emergence of floral silks until 10 days after pollination, when they were in senescent silk stage.

DISCUSSION

The ability to infect maize silks of plants inoculated before pollination and in senescent silk tissues in both periods confirmed the presence of *F. graminearum* colonies in APGA incubation. *F. graminearum* colonies detected in APGA confirmed the presence of *F. graminearum* which was observed under the microscope with maize silks clarified in lactophenol. This technique is a quantitative indicator of the presence of the pathogen in APGA, establishing an association between the host and the pathogen. Besides, this method resulted precise not only in identifying the presence of fungus but also in detecting the differences in hybrid behavior and moments of inoculation. Clarification technique is often used for extraction of embryos when smut is detected in wheat. However, this technique was used in this experiment to clarify and to observe *F. graminearum* hyphae in silks. Differences in silks incubated in APGA and infected with *F. graminearum* before pollination of both hybrids may be due to the presence of longer and more exposed silks towards the outer part of the ear, where fungus spores achieved a greater colonization of the silk in the hybrid PO1024. On the other hand, silks from the hybrid PO1170 had little exposure towards the outer part of the ear

Table 2. Incidence (%) of *F. graminearum* from maize silks of maize infected before pollination, in pollinated silks and in senescent silk tissues incubated on APGA (2005-2006).

Hybrids	Time of inoculation with *F. graminearum*			Means
	Before fecundation	During fecundation	Senescent stigma tissues	
PO1024	30 Aa	5 Ca	14 Ba	16
PO1170	6 Cb	7 Cb	16 Bb	10
Medias	18	6	15	

*Means followed by the same lower case letter in the column do not differ, and means followed by the same capital letter in the row do not differ at the 5% level (F test).

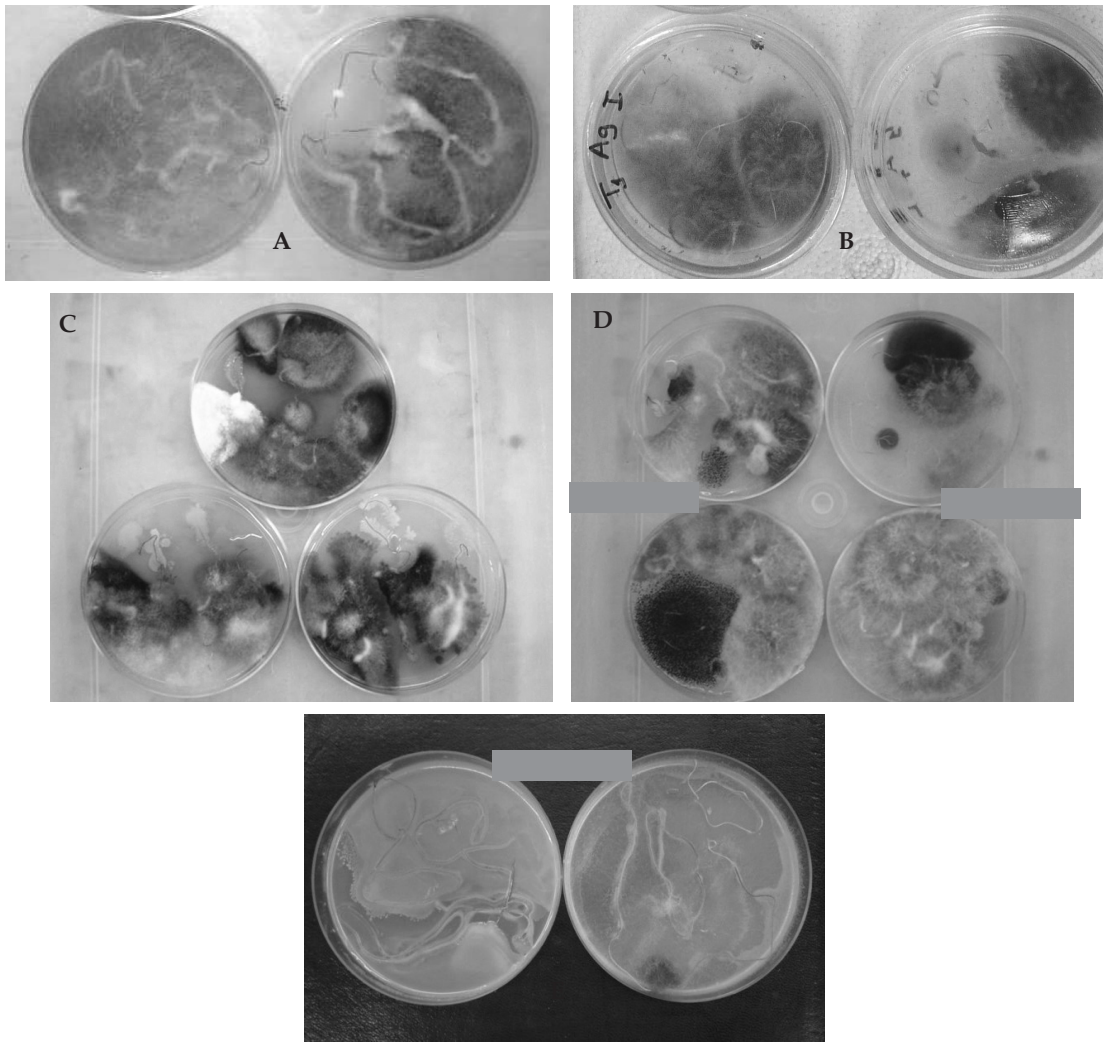

Fig. 6. Silks of maize flowers infected with *F. graminearum* colonies incubated in APGA of plants inoculated before and during pollination and in senescent silk tissues of PO1024 and PO1170 hybrids. Fig. 6 A and B show infected silks only by *F. graminearum* from ears inoculated before pollination. Fig. 6 C and D show colonies of *F. graminearum* isolated from ears inoculated during pollination and associated to senescent silk tissues, respectively. Floral silks used as controls (Fig. 6 E) presented only bacteria colonies. *Alternaria* spp., *Epicoccum* spp., *Aspergillus niger*, *A.flavus* and *Epicoccum* spp. were isolated as saprophytic and secondary (less important than *F. graminearum*) fungi from silk tissues during and after pollination (Fig. 6 C and D).

because this hybrid has a tight husk and is highly resistant, according to the results of this work, and also to White (1999) statement who considered that closed husk materials present a better behavior towards pathogens than open husk materials. Closed husks in the hybrid PO1170 would prevent the entry of the pathogen into the ear, while the hybrid PO1024 presents a loose husk and an apparent susceptibility to the entrance of the pathogen.

The lactophenol clarification technique used in the present study has allowed the observation of *F. graminearum* hyphae going from the inner part of the silk and across the conduction bundles and its constitutive cells. Also, dyeing permitted to differentiate active and senescent hyphae in silks of maize flowers. On the other hand, the incidence of active and senescent hyphae along the inoculated silks clarified in lactophenol was higher in plants inoculated before pollination and in senescent silk tissues than in plants inoculated during pollination. These results differed from those obtained by Reid et al. (1999), who reported the presence of *F. graminearum* during

pollination, approximately between the fourth and seventh day after silk emergence when silks elongated and were pollinated.

Silks are susceptible to fungus pathogen infections even when they were not pollinated. This shows that pollen grain, which takes part of fertilization process, would not be directly involved or could be a contributing factor of pathogen penetration. Additionally, silk biological age would not be a conditioning factor of infection, since some active hyphae were found in senescent silks in the present work. This result provides evidence that *F. graminearum* is a hemi-biotrophic phytopathogenic fungus. During inoculation with *F. graminearum* the main environmental conditions involved maximum temperatures between 29 and 30°C and a minimum relative humidity between 49 and 31% at every inoculation moment.

According to Reid et al. (1999) and Munkvold (2003), conditions that favor infection, growth and colonization of *F. graminearum* are temperatures between 24 and 26°C and high levels of relative humidity Therefore, the environmental conditions were suboptimal for the infection with this pathogen in this study. Ear rot caused by *F. graminearum* is favored by humidity in ears with 50% of exposed silks (R1), according to Ritchie scale during the period of silk growth. *F. graminearum* infection of maize silks at the three moments of inoculation presented in this study and under suboptimal environmental conditions showed the plasticity of this pathogen to adapt to unfavorable conditions and cause infections.

CONCLUSIONS

Silks are receptive *to F. graminearum* colonization along their complete length.

F. graminearum presence in senescent silk tissues shows the necrotrophic ability of this pathogen.

The hybrid PO1024 with open husks shows a greater susceptibility to pathogen attack than the hybrid PO1170 which presents closed husks.

F. graminearum infection in maize silks can occur before and after pollination and in senescent silk tissues.

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