



# Three inoculation methods for screening corn germplasm to white ear rot resistance

Justino L. Mário<sup>1</sup>, Erlei M. Reis<sup>2</sup> & Fernando C. Juliatti<sup>3</sup>

<sup>1</sup>Monsanto do Brasil Ltda, 38407-049, Uberlândia, MG, Brazil; <sup>2</sup>Universidade de Passo Fundo, 99052-900, Passo Fundo, RS, Brazil; <sup>3</sup>Universidade Federal de Uberlândia, 38400-902, Uberlândia, MG, Brazil

Author for correspondence: Fernando C. Juliatti, email: juliatti@ufu.br

## ABSTRACT

Three inoculation methods (natural, spraying and pouring) of *Stenocarpella maydis* in corn cobs were compared for their efficiency in screening corn genotypes for resistance to this pathogen. A total of six corn hybrids were inoculated with each method. Natural infection, spraying a conidial suspension on stigmas, or pouring it directly onto corn ears resulted in 21.02%, 39.78%, and 44.32% of infected ears. The high disease pressure provided by artificial inoculation of *S. maydis* allowed for a better differentiation between resistant and susceptible corn hybrids. The pouring technique was the easiest to carry out and better than the other methods under field conditions.

**Key words:** *S. macrospora*, *Stenocarpella maydis*, *Zea mays*, breeding.

## RESUMO

### Comparação de métodos de inoculação visando a seleção de germoplasma resistente à podridão branca da espiga do milho

Três métodos de inoculação de espigas de milho com *Stenocarpella maydis* (Berk) Sacc. possíveis de serem usados em programas de melhoramento foram testados em seis híbridos de milho. A infecção natural, a pulverização da suspensão conidial sobre os estigmas e a deposição da suspensão de esporos diretamente no pedúnculo resultaram em infecções médias de 21,02%, 39,78% e 44,32%, respectivamente. A maior intensidade da doença propiciada pelas inoculações por pulverização ou deposição permitiu melhor distinção entre híbridos resistentes e suscetíveis. O método de deposição foi estatisticamente superior aos demais, além de apresentar maior facilidade de execução em campo.

**Palavras-chave:** *S. macrospora*, *Stenocarpella maydis*, *Zea mays*, melhoramento.

## INTRODUCTION

One of the major constraints on increases in grain yields in corn crops in Brazil is the occurrence of stalk and ear rots, which damage both yield and grain quality (Pereira, 1995; Juliatti et al., 2007; Duarte et al., 2009). White cob rot or white ear rot (WER) occurs in practically all places where corn is cultivated (Clayton, 1927; Ullstrup, 1949; Thompson et al., 1971; Pereira & Pereira, 1976; Chambers, 1988; Bensch et al., 1992; Reis & Casa 1996; Dorrance et al., 1998). In Brazil the incidence of fungi of the genus *Stenocarpella* has increased due to changes in row spacing in corn fields, with growers now leaving 0.45 cm between lines (Juliatti et al., 2007; Duarte et al., 2009). In the Central and Southwestern regions of Brazil, stalk rot and WER are caused mainly by *Stenocarpella*. In the plateaus of Rio Grande do Sul, *S. macrospora* and *S. maydis* are frequently found causing seedling blight, stalk and ear rot (Casa, 1997), and *S. macrospora* has been associated with severe leaf spots (Mario & Prestes, 1997; Duarte et al., 2009). Genetic resistance seems to be the most promising and efficient way to control these pathogens (Klapproth & Hawk, 1991). However, fungicide sprays with triazols plus strobilurins or

triazols plus benzimidazols are the main procedure for disease control at the moment (Duarte et al., 2009).

To identify resistant germplasm, it is necessary to use reliable artificial inoculation methods (Ullstrup, 1949; Del Rio, 1990; Klapproth & Hawk, 1991; Bensch et al., 1992; Bensch, 1995). Methods of inoculation used in breeding programs should reproduce as closely as possible the infection under natural conditions. The selected method should also provide consistent data over the years, locations and genotypes, thus making it possible to define a clear distinction between susceptible and resistant genotypes (Ullstrup, 1970; Klapproth & Hawk, 1991; Del Rio & Melara, 1991; Bensch, 1995). Finally, these methods should be easy to apply.

This work aimed to compare methods of artificial inoculation for use in breeding programs, by screening inbred lines and hybrids resistant to WER and stalk rot.

## MATERIAL AND METHODS

### Experimental design

The experiment was conducted in a randomized block design and in split plot arrangements, with four

replications. Six hybrids constituted the main plot, and the methods of inoculation (natural, spraying and pouring) were the subplots. The experimental units consisted of two 5-meter lines, with 25 plants in each and a 0.8-meter distance between lines.

The experiments were conducted in the 1995/96 and 1996/97 growing seasons in the experimental unit of Braskalb, in Coxilha, RS, under a no-tillage system.

### Maize Hybrids

Six hybrids obtained from four seed companies were tested, three with grains of hard texture (AG9012, C808 and P3041) and three with soft texture (C901, XL212 and X9403). The choice of the hybrids was made on the basis of the described characteristics in the work of Klapproth & Hawk (1991).

### Isolation and production of inoculation

Four hundred grains collected from ears with typical WER symptoms were placed in a moist chamber (seven days at 25°C and 95% relative humidity) to stimulate the formation of pycnidia. Later, with the aid of a stereoscopic microscope and a histological needle, a pycnidium was removed from the grain, placed on a water drop and covered with a cover slip. The conidia were examined at 50x magnification for species identification. Once the species was identified, the conidia were transferred to a drop (approx. 1.0 mL) of sterile distilled water (SDW) placed on a plastic Petri dish containing potato-dextrose-agar (PDA), and spread on the surface of the substrate. The plates were incubated for three days at 23 to 27°C, the colonies were transferred to new Petri dishes with PDA and incubated for an additional three to four days at 25°C.

The substrate for inoculum production was prepared as follows. One hundred grams of sorghum grains in 1L Erlenmeyer flasks was washed in tap water, shaken by hand. The grains were absorbed in 125 ml of distilled water for approximately 12 hours; afterwards, the water not absorbed by the grains was discarded. After that, the substrate was autoclaved at 125°C for 20 minutes, and this operation was repeated twice. Five discs of a *S. maydis* colony, 5.0 mm in diameter, were transferred with the help of a histological needle, to an Erlenmeyer flask with the grain sorghum substrate. Flasks were then shaken to distribute the mycelial discs evenly in the substrate. The flasks were incubated at 25°C until a black mass of spores was formed around the grains, and were then maintained in a shaker for five days until uniform distribution was achieved. This procedure was carried out with a mixture of six isolates, one from each corn hybrid mentioned in the previous section.

### Inoculum preparation

The inoculum from one flask was suspended in 250 mL of SDW, shaken for 30 minutes, and transferred to another flask by filtration through five layers of cheese-

cloth, supported by a plastic funnel. Conidial concentrations were counted in a Neubauer chamber and the suspension was adjusted to  $4 \times 10^8$  conidia mL<sup>-1</sup>.

### Inoculation methods

Three inoculation methods were evaluated. The first was natural inoculum incidence from *Stenocarpella* spp. The second method was performed by spraying 5 mL of a spore suspension on the stigmas of the ears (Ullstrup, 1949; Klapproth & Hawk, 1991). After 10 days, 100% of the plants in the population had emitted the female flower. This method is useful because it reproduces natural inoculation (Ullstrup, 1949; Koehler, 1951). The third method consisted in pouring the spore suspension through the floral bracts on the peduncle with the help of an automatic dosing syringe (Incopelã brand, model 01, 50 mL). Five mL were poured on each ear, ten days after all the plants had flowered.

### Disease assessment

For disease assessment, ears in each treatment were harvested when grains reached between 18 and 24% of moisture (Anônimo, 1996). Three evaluation methods were used to quantify the disease: incidence of *Stenocarpella* spp. in ears, severity of *Stenocarpella* spp. in ears, and a grain health assay.

The incidence of *Stenocarpella* in ears was evaluated on the basis of the structures of *Stenocarpella* spp. (white mycelia and dark pycnidia) or symptoms in grains. To confirm *Stenocarpella* presence in the plots, samples from 10 ears were incubated at 25°C for 5 days at 95% RH until they developed a gray color and white mycelia with dark pycnidia. Healthy ears were visually separated from the infected ones. The incidence was expressed in percentage of infected ears.

To evaluate the severity of *Stenocarpella* spp. in the ears, infected ears were classified into four categories: 1) non-infected ear or ear without symptoms; 2) ear with up to 25% of infected area; 3) ear with between 25 and 50% of infected area; 4) ear with an infected area above 50%. The procedure of McKinney (1923) was applied to the number of ears in each category of severity in order to calculate the degree of severity (%) for each treatment.

For the grain health assay, a sample of 200 kernels per treatment, conditioned in cheese-cloth, was first washed in running tap water for 30 seconds, immersed in a solution of ethanol at 95%, and then immersed in a 2.0% sodium hypochlorite solution and shaken for three minutes. Then, in the flow chamber, the kernels were rinsed in 500 ml of SDW and dried on sterile filter-paper. Ten kernels were equidistantly placed in a germbox (experimental unit) lined at the bottom with three layers of filter-paper damp with SDW. Two hundred kernels per treatment were incubated, divided into four replications of 50 kernels. The boxes were incubated in a growth chamber at 25°C and 12 hours daylength until the differentiation of the color of *S. macrospora* and *S. maydis* colonies and pycnidia formed on the kernels, as described by Mario & Reis (2001) and Silva et al. (2005).

## Statistical analysis

Data were submitted to analysis of variance using arcsin square root transformation of means, because they did not follow a normal distribution according to Lilliefors' test. The transformed means were compared using Tukey's test at 5% probability in the Sannest computational program, version 7.0.

## RESULTS AND DISCUSSION

### Experiment of 1995/96

The spraying and pouring methods resulted in higher incidence of *S. maydis* in grains, and higher incidence and higher severity of *Stenocarpella spp* in ears. These results show that for the evaluation and selection of genetic material for resistance to WER, it is necessary to use artificial methods of inoculation, which is in agreement with what was previously reported by Ullstrup (1970), Correa (1978), Del Rio (1990), Klapproth & Hawk (1991), Del Rio & Melara (1991) and Bensch (1995), all of whom recommended the use of artificial methods of inoculation in corn breeding programs.

### Experiment of 1996/97

In this season, the three methods of inoculation presented different results. The pouring method presented the highest incidence, differing statistically from the other two. The spraying method presented an intermediate position, whereas natural infection presented the lowest intensity (Table 1). In this experiment, the spraying method presented a lower incidence than in the 1995/96 experiment. According to Koehler (1942) and Bensch et al. (1992), this method is influenced by weather conditions, so it should be used with caution. In contrast, Klapproth & Hawk (1991) and Silva et al. (2005) recommended this method.

### Incidence of *S. maydis* in grains

Hybrid X9403 presented the lowest incidence of the target pathogen in grains under the tested methods. The other hybrids presented a variable incidence according to

the method (Table 2). Two hybrids changed their reactions according to the inoculation method: XL212 presented a higher incidence of *S. maydis* when inoculated by spraying compared to the other methods, while a higher incidence of the pathogen was observed in AG9012 when this hybrid was inoculated by the pouring method. No influence of these two methods of inoculation on the reaction of the other hybrids was observed. Our results are in disagreement with those from Klapproth & Hawk (1991), who reported spraying inoculum suspension on the stigmas to be the best method for use in breeding programs for the selection of materials with resistance to WER.

### Incidence of *Stenocarpella spp.* in ears

Maize hybrids XL212 and X9403 had the lowest incidence of the pathogen on the ears regardless of the method of inoculation (Table 2). The fact that these two hybrids have dented (soft) grain reinforces previous results obtained by Correa (1978), who found that 70% of dented germplasm presented a lower incidence of *S. maydis* in the ears when compared to other harder germplasm. In the pouring method, the hybrid AG9012 presented a significantly higher incidence, and the hybrids C901, C808 and P3041 were in an intermediate position. These results are similar to those reported by Flett & McLaren (1994). These authors stated the need for a minimum of 17% incidence in the ear to differentiate resistant germplasm from susceptible one.

### Severity of *Stenocarpella spp.* in ears

These results were similar to those of the evaluations of disease incidence. Hybrids XL212 and X9403 showed significantly lower disease severity than the others (Table 2). It may be inferred that there are differences in the reaction of cultivars considering resistance to WER. Nevertheless, our data are not enough to recommend the tested hybrids as resistant under natural field conditions. In field plots without artificial inoculum it is also possible to find a false reaction on the ears (Ullstrup 1970; Silva et al., 2005).

**TABLE 1** - Comparison of inoculation methods of *Stenocarpella* on the incidence and severity of *Stenocarpella spp.*

Methods	Incidence in grains (%)	Incidence in ears (%)	Severity in ears (%)
	<i>S. maydis</i>	<i>Stenocarpella spp.</i>	<i>Stenocarpella spp.</i>
		<b>1995/96</b>	
Natural infection	3.25 b	33.31 b	17.65 b
Pouring	46.07 a	61.85 a	35.81 a
Spraying	47.05 a	60.72 a	34.25 a
		<b>1996/97</b>	
Natural infection	12.03 c	39.58 c	20.61 c
Pouring	30.32 a	60.23 a	31.68 a
Spraying	19.82 b	50.16 b	26.73 b
CV (%)	23.02	9.10	8.40
DMS 5%	4.61	2.86	1.83
Prob > F	0.007	0.001	0.001

Means followed by the same letter in the columns do not differ statistically according to Tukey's multiple range test at  $p = 5\%$ .

**TABLE 2** - Comparison of inoculation methods on the incidence and severity of *Stenocarpella maydis* and *S. macrospora* in corn grain and ears

Hybrids	Methods		
	Natural infection	Spraying	Pouring
	<i>S. macrospora</i>	Incidence of <i>S. maydis</i> in grains (%) <sup>1</sup>	
AG9012	8.50 ab C	22.11 bc B	54.31 a A
C901	10.58 a B	42.78 a A	52.28 ab A
P3041	4.73 ab B	44.16 a A	46.17 ab A
C808	6.75 ab B	36.28 ab A	37.49 b A
X9403	1.83 b B	16.09 c A	21.38 c A
XL212	5.05 ab C	37.99 a A	20.02 c B
	<i>S. macrospora</i>	Incidence of <i>Stenocarpella</i> spp. in ears (%) <sup>2</sup>	
AG9012	44.62 a C	61.58 a B	81.94 a A
C901	38.70 a B	66.30 a A	71.68 b A
C808	43.92 a B	60.37 a A	65.00 b A
P3041	41.01 a B	62.24 a A	62.73 b A
X9403	24.67 b B	36.41 b A	43.33 c A
XL212	26.85 b B	45.57 b A	38.42 c A
	<i>S. macrospora</i>	Severity of <i>Stenocarpella</i> spp. in ears (%) <sup>3</sup>	
C901	20.85 a B	39.05 a A	42.96 a A
AG9012	23.16 a C	33.11 a B	42.83 a A
C808	23.66 a B	36.82 a A	37.81 a A
P3041	20.79 a B	36.33 a A	36.34 a A
X9403	13.75 b B	19.42 b A	21.28 b A
XL212	13.54 b C	23.94 b A	19.07 b B

<sup>1</sup>CV = 23.0%; DMS 5% = 9.45; <sup>2</sup>CV = 9.1%; DMS 5% = 6.96; <sup>3</sup>CV = 8.4%; DMS 5% = 4.05. Means followed by the same lower-case letters in the column and upper-case letter in the line do not differ statistically according to Tukey's multiple range test at  $p = 5\%$ .

### Natural incidence of *S. macrospora* in corn grains

The natural infection of corn grains by *S. macrospora* was not influenced by any of the artificial inoculation methods. Therefore, no significant difference was observed between treatments regarding the incidence of the pathogen (Table 3).

In the 1996/97 experiment, the natural incidence of *S. macrospora* in the corn grains was twice as that of the 1995/96 growing season. Other authors have confirmed the presence of this pathogen under field conditions in grains (Mora & Moreno, 1984 and Del Rio, 1990) or natural inoculum sources such as crop residues in corn monoculture (Mario & Reis, 2003).

Overall, the pouring method lead to the highest incidence of the disease, and therefore this method allowed us to clearly distinguish the susceptible germplasm from

the resistant one, showing that its use in the selection of resistant materials to WER is possible (Bensch et al., 1992, Silva et al, 2005). This method seems to be hardly influenced by climatic oscillations. Based on these results the pouring method can to be recommended for breeding programs and germplasm screening to select genotypes and populations (Silva et al, 2007) for resistance to ear rot by *S. maydis*. Using this method, both incidence and severity of *S. maydis* can be used as variables for germplasm screening to resistance against the pathogen under field conditions.

### REFERENCES

- Alves MIF et al. (1993) Tópicos especiais de estatística experimental utilizando o SANEST. Anais da 5ª Reunião Anual da Sociedade Internacional de Biometria. Porto Alegre RS. UFRGS.
- Anônimo (1996) Recomendações técnicas para a cultura do milho no estado do Rio Grande do Sul. Porto Alegre RS. FEPAGRO.
- Bensch MJ (1995) An evaluation of inoculation techniques inducing *Stenocarpella maydis* e ear rot on maize. Journal of Plant and Soil 12:172-17
- Bensch MJ, Van Staden J, Rijkenberg JHF (1992) Time and site of inoculation of maize for optimum infection of ears by *Stenocarpella maydis*. Journal of Phytopathology 136:265-269
- Casa RT (1997) *Diplodia maydis* e *D. macrospora* associadas à semente de milho. Dissertação de Mestrado. Universidade Federal de Viçosa. Viçosa MG.

**TABLE 3** - Natural incidence of *Stenocarpella macrospora* in corn grains, in plants inoculated with *S. maydis*

Methods	Incidence of <i>S. macrospora</i> (%)	
	1995/96	1996/97
Natural infection	12.36 a	23.82 a
Pouring	13.90 a	22.66 a
Spraying	10.70 a	22.97 a

CV = 12.77%; DMS 5% = 3.25. Means following by the same letters in the columns do not differ statistically according to Tukey's multiple range test at  $p = 5\%$ .



- Chambers KR (1988) Effect of time of inoculation on *Diplodia* stalk and ear rot of maize in South Africa. *Plant Disease* 72:529-531
- Clayton EE (1927) *Diplodia* ear rot disease of corn. *Journal of Agricultural Research* 34:357-371
- Correa CFP (1978) Métodos de inoculação e fontes de resistência para *Diplodia maydis* (Berk) sacc. e *Fusarium moniliforme* Sheldon, agentes causadores de podridões de espigas de milho (*Zea mays* L.). Dissertação de Mestrado. ESALQ-USP. Piracicaba SP.
- Del Rio L, Melara W (1991) Evaluacion de la resistencia a maiz muerto de algunos híbridos y variedades de maiz comunes en Honduras. *Ceiba*, v. 32, p.127-132
- Del Rio L (1990) Maiz muerto en Honduras provocado por el complejo *Diplodia* y *Fusarium*. *Manejo Integrado de Plagas* 18:42-53
- Dorrance AE, Hinkelman KH, Warren HL (1998) Diallel analysis of *Diplodia* ear rot resistance in maize. *Plant Disease* 82:699-703
- Duarte RT, Juliatti FC, Freitas PT (2009) Eficácia de diferentes fungicidas na cultura do milho. *Bioscience Journal* 25:101-111
- Flett BC, McLaren NW (1994) Optimum disease potential for evaluating resistance to *Stenocarpella maydis* ear rot corn hybrids. *Plant Disease* 78:587-589.
- Juliatti FC, Zuza JMLF, Souza PP, Polizel AC (2007) Efeito do genótipo de milho e da aplicação foliar de fungicidas na incidência de grãos ardidos de milho. *Bioscience Journal* 23:34-41
- Klapproth CJ, Hawk AJ (1991) Evaluation of four inoculation techniques for infecting corn ears with *Stenocarpella maydis*. *Plant Disease* 75:1057-1060
- Koehler B (1942) Natural mode of entrance of fungi into corn ears and some symptoms that indicate infection. *Journal of Agricultural Research* 64:421-442
- Mario JL, Prestes AM (1997) Avaliação da resistência à mancha foliar causada por *Diplodia macrospora* em genótipos de milho. *Fitopatologia Brasileira* 22(Supl.):280
- Mario JL, Reis EM (2001) Método simples para diferenciar *Diplodia macrospora* de *D. maydis* em testes de patologia de sementes de milho. *Fitopatologia Brasileira* 26:670-672
- Mario JL, Reis EM (2003) Quantificação do inóculo de *Diplodia macrospora* e de *D. maydis* em restos culturais, no ar e sua relação com a infecção em grãos de milho. *Fitopatologia Brasileira* 28:143-147
- McKinney HH (1923) Influence of soil temperature and moisture on infection of wheat seedlings by *Helminthosporium sativum*. *Journal of Agricultural Research* 26:195-217
- Mora LE, Moreno RA (1984) Cropping pattern and soil management influence on plant diseases: I. *Diplodia macrospora* leaf spot of maize. *Turrialba* 34:35-40
- Pereira OAP (1995) Situação atual de doenças da cultura do milho no Brasil e estratégias de controle. In: *Resistência Genética de Plantas a Doenças*. Piracicaba SP. ESALQ-USP
- Pereira OAP, Pereira WSP (1976) Estudo de *Diplodia zae* (Shw.) Lev. e *Fusarium moniliforme* (Sheldon) em colmos de milho. *Summa Phytopathologica* 2:157-165
- Pinto NJFA (2010) Cultivo do milho – Qualidade sanitária dos grãos. <http://sistemasdeproducao.cnptia.embrapa.br/FontesHTML/Milho/CultivodoMilho/dsanitaria.htm>. Access on 08/21/2010.
- Reis EM, Casa RT (1996) Manual de identificação e controle de doenças de milho. Passo Fundo RS. Aldeia Norte Editora.
- Silva AR, Juliatti FC, Brito CH, Gomes LS (2005) Métodos de inoculação de *Stenocarpella maydis* em três populações de milho. *Summa Phytopathologica* 31:79-83
- Thompson DL, Villena WL, Maxwell JD (1971) Correlation between *Diplodia* stalk and ear rot of corn. *Plant Disease Reporter* 55:158-162
- Ullstrup AJ (1949) A method for producing artificial epidemics of *Diplodia* ear rot. *Phytopathology* 39:93-101
- Ullstrup AJ (1970) Methods for inoculating corn ears with *Gibberella zae* and *Diplodia maydis*. *Plant Disease* 54:658-662

---

TPP 106 - Received 13 April 2011 - Accepted 5 December 2011  
Section Editor: Carlos R. Casela