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# Effects of temperature, light regime and substrates on the production and germination of *Stenocarpella maydis* pycnidiospores

**Paulo Roberto Kuhnem Júnior[1*], Ricardo Trezzi Casa[1], Amauri Bogo[2], Lenita Agostineto[1], Jonathan Marcel Bolzan[1] and David José Miqueluti[1]**

*[1]Departamento de Agronomia, Centro de Ciências Agroveterinárias, Universidade do Estado de Santa Catarina, Lages, Santa Catarina, Brazil. [2]Secretaria de Cooperação Institucional e Internacional, Universidade do Estado de Santa Catarina, Av. Madre Benvenuta, 2007, 88035-001, Florianópolis, Santa Catarina, Brazil. *Author for correspondence. E-mail: prkuhnem@gmail.com.*

**ABSTRACT.** This study aimed to evaluate the production and germination of *Stenocarpella maydis* pycnidiospores under in vitro conditions as affected by substrate composition (sorghum, wheat, black oat or barley), light regime (continuous dark, 12-h light dark$^{-1}$ or continuous light conditions), and incubation temperature (21, 24, 27, 30 or 33ºC). Each substrate (20 g of grain) was soaked in 100 mL of water for 24h and sterilized twice for 20 min at 127ºC. Three plugs (5 mm diameter) of a single-spored culture of *S. maydis* were used as inocula for each substrate. Assessments of pycnidiospore production per gram of grain and percent germination were made 14 days after inoculation. Barley, black oat or wheat grains were the best substrates for the mass production of *S. maydis* pycnidiospores and the maintenance of high germination rates. The highest pycnidiospore production (67,600 pycnidiospores g$^{-1}$) was obtained using barley grain as a substrate with incubation at 27ºC under a 12-h light dark$^{-1}$ cycle.

**Keywords:** *Diplodia*, ear rot, stalk rot, natural medium.

## Efeito de temperatura, regime de luminosidade e substratos sobre a produção e germinação de picnidioporos de *Stenocarpella maydis*

**RESUMO.** A produção e a germinação de picnidiosporos de *Stenocarpella maydis* foram avaliadas *in vitro* sob a influência da composição de substratos (sorgo, trigo, aveia preta e cevada), regime de luminosidade (escuro contínuo, fotoperíodo de 12h ou luz contínua) e temperatura de inbucação (21, 24, 27, 30 ou 33ºC). Três discos de 5 mm de cultura monospórica de *S. maydis* foram inoculados em cada substrato. A produção e o percentual de germinação de picnidiosporos foram avaliados aos 14 dias após a inoculação. Grãos de cevada, aveia preta e trigo foram os melhores substratos na produção e germinação de picnidiosporos de *S. maydis*. A maior concentração (67.600 picnidiosporos g$^{-1}$) foi obtida em grãos de cevada, à 27ºC e 12h de fotoperíodo.

**Palavras-chave:** *Diplodia*, podridão de grãos, podridão de colmo, meio natural.

## Introduction

*Stenocarpella maydis* (Berk.) Sutton [Syn. *Diplodia maydis* (Berk.) Sacc.] is an important fungal pathogen of maize (*Zea mays* L.) and causes both ear and stalk rots (WHITE, 1999). These diseases are distributed worldwide, and in Brazil, they are most prevalent in areas with no-tilled monoculture systems (CASA et al., 2006; ZAMBOLIM et al., 2000). The pathogen survives on maize plant debris and internally in seeds as pycnidia or mycelium (CASA et al., 1998, 2003). Infected ears can cause kernel rot, which downgrades production values for the maize industry (REIS et al., 2004).

In maize breeding programs, pycnidiospore suspensions are mostly used as inocula in experiments to assess the resistance to disease induced by *S. maydis* under conditions of natural infection or artificial inoculation in the field (ANDERSON; WHITE, 1994; KOEHLER, 1951; MÁRIO; REIS, 2003; PAPPELIS et al., 1973). For the latter, a large amount of inoculum may be necessary (MÁRIO; REIS, 2003; ULLSTRUP, 1970).

Previous research has shown that the viability of pycnidiospores decreases with continued exposure to sunlight (BRUNELLI et al., 2006), and polysaccharides such as dextrose but not cerelose are required for growth (KINSEL, 1937). Species of the *Stenocarpella* genus usually present fast mycelial growth but reduced sporulation efficiency on artificial media (WHITE, 1999). Culture media such as PDA (potato + dextrose + agar) (MORANT et al., 1993), PSA (potato + sucrose + agar) (CASA et al., 2007), OFA (oat flour + agar) (JONG; EDWARDS,

1991) and malt extract-yeast + agar (BIZZETTO et al., 2000) have been used for *Stenocarpella* mycelial production but result in very low sporulation rates. Morant et al. (1993) observed that adding biotin to the media has a stimulatory effect on *S. macrospora* sporulation.

In addition to culture media, environmental factors such as temperature and light exposure are essential for fungal sporulation (DHINGRA; SINCLAIR, 1995). According to Griffin (1994), proteins and enzymes that are responsible for fungus cell maturation are stimulated by temperature. However, light exposure and light quality could inhibit the reproductive capacities of some fungal cells (HAWKER, 1957).

Many substrates that are based on oat, rice, barley, corn, soybean and sorghum grains are reported to improve the mycelial growth rates of many different fungi on artificial media (DHINGRA; SINCLAIR, 1995; HARTMAN et al., 1997; KLINGELFUSS et al., 2007; MELGAR; ROY, 1994; TOLEDO et al., 2004). Although *Stenocarpella* spp. has been grown on maize stalks (CASA et al., 2007), sorghum grains (ULSTRUP, 1970) and oat grains (MORANT et al., 1993), pycnidiospore production was poor, and the incubation period was longer than 30 days on these substrates.

In plant pathological research, the development of affordable and efficient methods to produce abundant, uniform inocula for artificial inoculations is key. Hence, the objective of this study was to develop such a method by evaluating the effect of natural substrates, temperature and light regimes on the production and germination of *S. maydis* pycnidiospores under *in vitro* conditions.

## Material and methods

Experiments were carried out at the Centro de Ciências Agroveterinárias (CAV), UDESC (Universidade do Estado de Santa Catarina). Maize kernels infected with *S. maydis* were obtained in a commercial field located in the Abelardo Luz municipality, Santa Catarina State, Brazil. Kernels were disinfested in 1% NaOCl and plated on potato-dextrose agar (PDA). Plates were maintained under cool, white fluorescent lamps (Philips 15W/75) at 25°C for 5 days until mycelial growth reached approximately 3.0 cm in diameter. A virulent *S. maydis* isolate (SM1) from the CAV culture collection that was tested in previous studies was used in the experiment.

A factorial experiment with three factors, substrate (sorghum, wheat, black oat or barley grains), light regime (continuous dark, 12-h light dark$^{-1}$ cycle or continuous light conditions), and temperature (21, 24, 27, 30 or 33°C) was designed to evaluate the mass production and percentage of germination of *S. maydis* pycnidiospores under *in vitro* conditions.

Twenty grams of each substrate were soaked with 100 mL of sterilized water in 1 L Erlenmeyer flasks for 24h, after which time the excess water was removed, and the substrates were sterilized twice for 25 min. at 127°C in intervals of 24h. Three plugs (5 mm in diameter) of mycelial growth from PDA medium containing hyphal tips of *S. maydis* were used to inoculate the substrates, which were incubated for 14 days (FLETT; McLAREN, 2001) for maximum pycnidiospore production. Every 5 days, the flasks were rotated manually and gently for 10 s to homogenize the substrates. Pycnidiospores were harvested by combining 20 mL of distillated water plus 0.05% of Tween-10 and 2 g of the contents of each flask in a 50 mL tube and homogenizing the mixture for 10 s in a vortex at high speed. Four 0.03 mL aliquots of each spore suspension tube were deposited onto a microscope slide. The pycnidiospore concentration per slide was determined using an optical microscope (40 x objective) and converted to the number of spores per gram of substrate. Pycnidiospore germination was determined on a volume of 1 mL of spore suspension in 1% water-agar plates that were incubated for 6h in BOD (biochemical oxygen demand) continuous light systems at 25°C (CASA et al., 2007), and the percentage of pycnidiospore germination was assessed directly on the plates using an optical microscope (40x objective). A total of 50 pycnidiospores per plate were counted, and the percentage of pycnidiospores showing germ tubes with twice the diameter of the spore, which were considered germinated, was assessed (CASA et al., 2007).

The experiments followed a completely randomized design. The linear model analysis of covariance (ANCOVA) was used to investigate the effects of single and interaction responses of the factors. The two response variables, number of pycnidiospores g$^{-1}$ and percentage of pycnidiospore germination, were square root-transformed to normalize the distribution. The comparison of the average value for levels of substrate and light regime were performed using a Bonferroni Test, and the temperature of each substrate and light regime combination was assessed by polynomial regression (STEEL et al., 1997). The statistical analysis of the data was performed by WinStat software (MACHADO; CONCEIÇÃO, 2009), and all tests that were performed considered the minimum

level of 1% of the significance bounds range distribution (p = 0.0001).

## Results and discussion

*Stenocarpella maydis* pycnidiospores were produced in all substrates, temperature and light regimes evaluated in this study. However, there were significant effects of light regime, temperature, substrate and their interactions, including a triple interaction, on pycnidiospore production (Table 1).

*Stenocarpella maydis* responded differently to the qualitative factors of substrate and light regime for pycnidiospore production and percentage of germination (Table 2).

**Table 1.** Analysis of covariance (ANCOVA) of pycnidiospore production $g^{-1}$ and percentage of germination of *Stenocarpella maydis* for light regime, temperature and substrate.

| Number of factors (sources) | Degrees of freedom | Mean Square | |
|---|---|---|---|
| | | Spore production $g^{-1}$ | Germination (%) |
| Light regime (L) | 1 | 77,670.2* | 44.4* |
| Temperature (T) | 4 | 65,552.5* | 23.8* |
| Substrate (S) | 3 | 27,814.9* | 4.4* |
| L x T x S | 12 | 1,499.3* | 1.3* |
| Error | 231 | 878.9 | 0.5 |
| Total | 251 | - | - |
| C.V | - | 28.72 | 19.29 |

*Significance level for *F* test (p = 0.0001); C.V.- Coefficient of variance.

**Table 2.** Production and percentage of germination of *S. maydis* pycnidiospores for light regime, temperature and substrate.

| Light regime | Substrate | Spore production $g^{-1}$ | Germination (%) |
|---|---|---|---|
| 12-h light dark $cycle^{-1}$ | - | 5,900 a | 55 a |
| Continuous light | - | 5,700 a | 57 a |
| Continuous dark | - | 660 b | 24 b |
| - | barley | 11,800 a | 60 a |
| - | black oat | 4,100 b | 48 b |
| - | wheat | 4,100 b | 47 b |
| - | sorghum | 2,700 c | 43 c |

*The same letters do not differ by the Bonferroni test (p = 0.0001).

A non-significant difference was observed between the 12-h light $dark^{-1}$ and continuous light regimes on pycnidiospore production and the percentage of germination (Table 2). Lower levels of pycnidiospore production and percentage of germination were observed under continuous dark conditions regardless of the temperature or substrate (Table 2). However, the largest numbers of pycnidiospores $g^{-1}$ (11,800) and 60% germination efficiency were obtained with the barley grain substrate regardless of the light regime or temperature (Table 2).

The adjusted polynomial regression of all substrates for pycnidiospore production and percentage of germination were significant (p = 0.0001), with the exception of pycnidiospore germination on a wheat substrate with a 12-h light $dark^{-1}$ cycle and continuous light and a black oat substrate with a 12-h light $dark^{-1}$ cycle (data not shown).

The barley grain substrate at 27ºC under a 12-h light $dark^{-1}$ cycle yielded the largest number of pycnidiospores per gram (67,600), 93% of which germinated after 14 days of incubation; therefore, these conditions are recommended for mass inoculum production (Figures 1 and 2).

Hanada et al. (2002) and Brunelli et al. (2006) observed that *Mycosphaerella fijiensis* (Morelet) Deighton and *Cercospora zeae-maydis* Tehon & Daniels did not produce conidia in the absence of light. Light exposure has a direct effect on the activation of key enzymes of spore production (MACRAE; YODER, 1988). Nevertheless, some fungal species do not seem to respond to light exposure (PAUL; MUNKVOLD, 2005).

Data from this study corroborate many other reports (BRUNELLI et al., 2006; HANADA et al., 2002; MAFACIOLI et al., 2008; NOZAKI et al., 2004; ULLSTRUP, 1970) in which a temperature between 22 and 27ºC was found to be the optimum interval for inoculum production. Minimum and maximum temperatures of 21 and 33ºC, respectively, resulted in lower *S. maydis* pycnidiospore production (Figure 1) and percentage of germination (Figure 2) for all substrates tested.

In contrast to our results, Casa et al. (2007) reported a 30% increase in *S. macrospora* conidia germination on PSA (potato-sucrose-agar) under continuous light compared to continuous dark conditions. The barley grain substrate was best suited for the mass production of pycnidiospores; under these conditions, the pycnidiospores retained 93% of their germination capability. This value is higher than the value found by Morant et al. (1993), who reported *S. maydis* pycnidiospore germination percentages of 86% and 96% on an oak grain substrate and mineral salt medium, respectively.

When studying mycelial growth and sporulation of *S. maydis* on sorghum, oak and agar-based substrates, Bizzetto et al. (2000) obtained no more than 10,000 pycnidiospores $mL^{-1}$ after 15 days of incubation. Previously, Ullstrup (1970) obtained 1,200,000 pycnidiospores $mL^{-1}$ of *S. maydis* after 6 weeks of incubation at 24ºC under a 12-h light $dark^{-1}$ cycle. In contrast, a barley grain substrate at 27ºC under a 12-h light $dark^{-1}$ cycle yielded approximately 700,000 pycnidiospores $g^{-1}$ after 14 days of incubation, which is slightly more than half of the number produced using Ullstrup's (1970) method; however, these spores numbers were achieved in a significantly shorter time, demonstrating the efficiency of the present method to produce viable pycnidiospore inocula for experimental research.

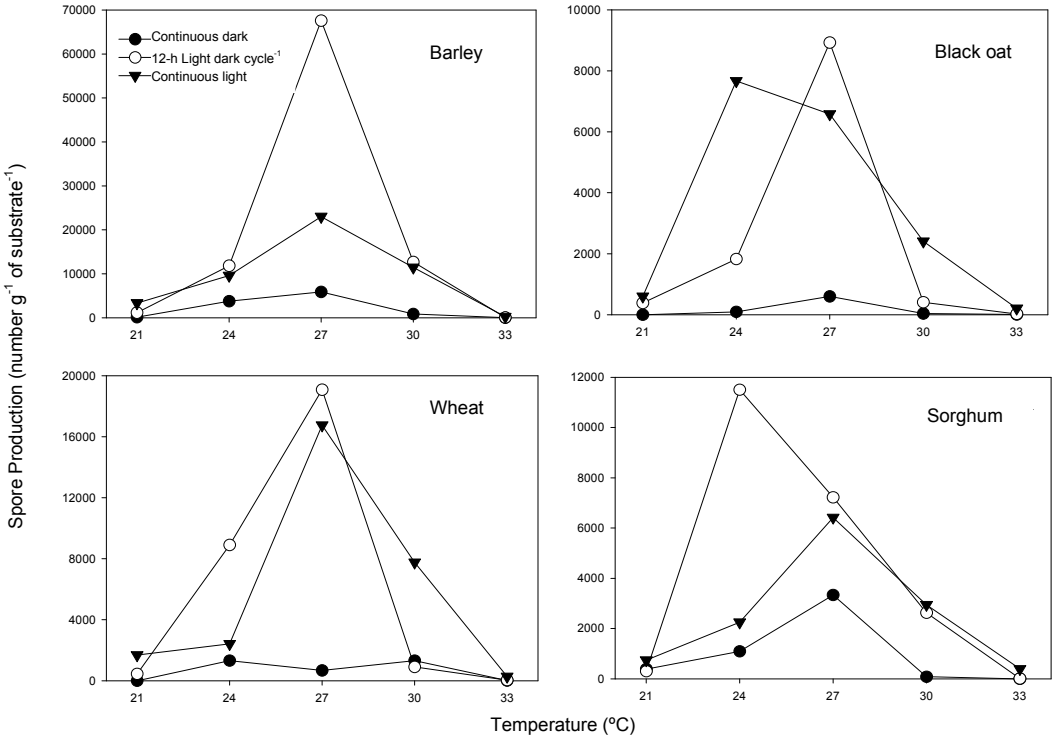


**Figure 1.** Pycnidiospore production (number per gram of substrate) in substrates and temperature for continuous dark, 12-h light dark$^{-1}$ cycle and continuous light conditions. The ANCOVA performed on the square root-transformed data showed a significant ($F_{0,001}$ = 60.21) effect of the interaction 'substrate x temperature x light regime,' with BON $_{0,001}$ = 14.01. The data in the graph are the original (non-transformed) values.

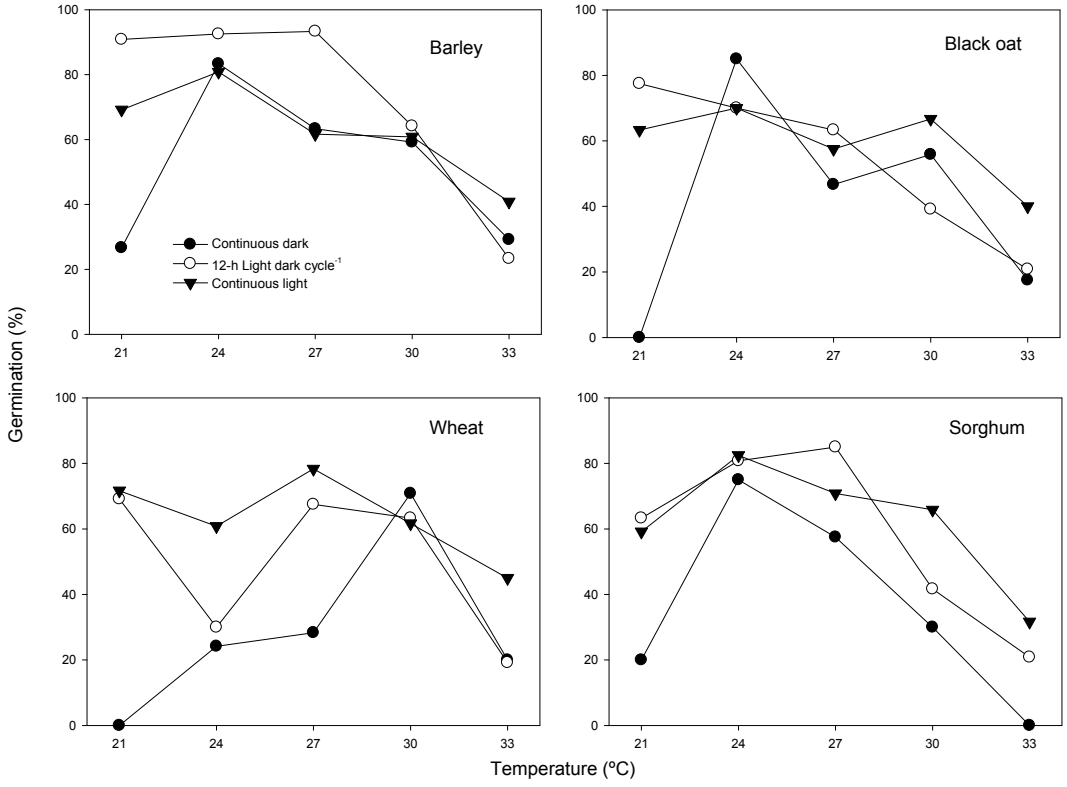


**Figure 2.** Pycnidiospore germination (%) in substrates and temperature for continuous dark,12-h light dark$^{-1}$ cycle and continuous light conditions. The ANCOVA performed on the square root-transformed data showed a significant ($F_{0.001}$ = 21.53) effect of the interaction 'substrate x temperature x light regime,' with BON $_{0.001}$ = 3.01. The data in the graph are the original (non-transformed) percentage values.

## Conclusion

The production of large numbers of *S. maydis* pycnidiospores while maintaining a high germination rate can be achieved using barley grain as a substrate and incubation at 27ºC under a 12-h light dark$^{-1}$ cycle. Light was found to be an essential factor for the asexual reproduction of this fungal species.

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