A Synthetic Medium for Mass Production of Pycnidiospores of *Stenocarpella* Species

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ABSTRACT

Morant, M. A., Warren, H. L., and von Qualen, S. K. 1993. A synthetic medium for mass production of pycnidiospores of *Stenocarpella* species. Plant Dis. 77:424-426.

Sporulation and vegetative growth of *Stenocarpella maydis* and *S. macrospora* were evaluated by growing the organisms in basal mineral salts medium amended with sucrose (0–30 g/L) and biotin (0–50 mg/L) for 28 days at 23 C under constant illumination. Sucrose at 5 or 10 g/L and biotin at 6 or 8 mg/L yielded the largest quantity of pycnidiospores of each organism. Sporulation of *S. maydis*, however, was higher than that of *S. macrospora* at all concentrations of biotin, although 6 mg/L gave the largest number of spores compared with biotin-less medium. Pycnidiospore concentrations of *S. maydis* and *S. macrospora* in media amended with 10 g/L of sucrose and 6 mg/L of biotin were 0.73×10^6 and 0 per milliliter, respectively. In the absence of biotin, pycnidiospore concentrations were 3.0×10^6 and 0.31×10^6, respectively. Production of mycelia was enhanced by biotin but was independent of its concentration, and there was an inverse relationship between vegetative growth and sporulation for each organism.

Additional keyword: corn

Stenocarpella maydis (Berk.) Sutton (syn. *Diplodia maydis* (Berk.) Sacc.) and *S. macrospora* (Earle) Sutton (*D. macrospora* Earle) are fungal pathogens of maize (*Zea mays* L.) that cause seedling blight and root, ear, and stalk rots. *S. macrospora* incites Diplodia leaf streak, which occurs in the southern United States and is most prevalent in tropical and subtropical areas (5). Although maize and its ancestor, teosinte, (1–3) are the only known hosts of these organisms, the diseases caused by *Stenocarpella* species are distributed worldwide, with *S. maydis* being more prevalent in the United States. Annual yield reduction in the United States corn belt due to stalk and ear rot pathogens, including *S. maydis*, can exceed 10^9 bushels (8).

The pathogens, which survive in plant debris and on seeds, are most easily distinguished by the sizes of their pycnidia and pycnidiospores (2,3). Optimum temperature for growth is 18–24 C (2,3). Viability of pycnidiospores decreases with continued exposure to sunlight (2,3). Kinsel (4) reported that polysaccharides were required for growth of *S. macrospora*, while Stevens and Larsh (12) specified that it could grow on cane sugar but not on Cerelose (commercial corn sugar). *S. maydis*, on the other hand, grew on Cerelose but not on cane sugar. The ability of the organisms to utilize these carbohydrates was later reported to depend on availability of biotin or a biotinlike substance (6). This has been confirmed for *S. macrospora* but not for *S. maydis* (2,3).

To conduct research involving these organisms, scientists of various disciplines frequently require large numbers of pycnidiospores of similar physiological maturity. When germ plasm is being evaluated for resistance, pycnidiospore suspensions are used as inoculum. Generally, inoculum is produced by growing the organisms on potato-dextrose agar, organic substrates (oat, wheat, or a combination of these substrates), or other synthetic media (1,7). Some reports, however, indicate that cultural conditions result in morphological variations (9,13) and could also conceivably influence the physiology of the pycnidiospores and initiation of the disease process.

For uniformity of tests among researchers, a standard medium that yields abundant, morphologically, physiologically, and chronologically uniform pycnidiospores is needed. The present study was undertaken to formulate a culture medium suitable for large-scale production of inoculum of *S. maydis* and *S. macrospora*.

MATERIALS AND METHODS

Three maize kernels infected with *S. maydis* and three infected with *S. macrospora* were disinfested in 1% NaOCL and plated on potato-dextrose agar. Infected kernels were obtained from Purdue University's Agronomy Farm in West Lafayette, Indiana. Plates were maintained under constant cool-white fluorescent lamps with a light intensity of 50 μE at 23 C for 4 days until mycelial growth from each kernel was about 2.5 cm in diameter. One plug (5 mm in diameter) of medium, including hyphal tips, from each colony was subsequently used to inoculate modified basal mineral salts medium (10). The medium contained 2.65 g of $(NH_4)_2SO_4$, 2.38 g of KH_2PO_4, 5.6 g of $K_2HPO_4 \cdot 3H_2O$, 1.0 g of $MgSO_4 \cdot 7H_2O$, and 1.0 ml of Pridham and Gottlieb trace salts solution per liter of glass-distilled deionized water. This medium was selected from the 12 media screened because it gave the lowest yield of mycelia (H. L. Warren, *unpublished*). It was amended with sucrose ranging from 0 to 30 g/L in 5-g increments. Biotin was also added at concentrations ranging from 0 to 10 mg/L in 2-mg increments, then to 50 mg/L in 10-mg increments.

Each ingredient was dissolved separately before being combined, and the final volume was adjusted to 1 L with glass-distilled deionized water. Then, 200 ml of medium per 1-L Erlenmeyer flask was stoppered with a cotton plug and sterilized at 15 psi and 121 C for 20 min. After the medium cooled to ambient temperature, sterile trace salt and biotin solutions were added aseptically. The flasks were inoculated with a 5-mm-diameter mycelial plug of hyphal tips, then maintained under conditions similar to those used for mycelial production for 28 days to achieve maximum sporulation. Pycnidiospores were harvested by combining 100 ml of distilled water and the contents of a flask in a commercial blender and homogenizing the mixture for 30 sec at high speed. Pycnidiospore concentration was determined with a hemacytometer.

The experiment was conducted as a completely random design with three replications for each species and each of the three colonies. The test was repeated three times, and the values were averaged. Data were analyzed statistically with the Statistical Analysis System (SAS Institute, Cary, NC), and significant differences between means were estimated with Duncan's multiple range test.

Accepted for publication 11 January 1993.

Percent pycnidiospore germination of the two organisms was also compared on oat culture and basal mineral salts medium. Oat seeds were autoclaved at 121 C and 15 psi on each of two consecutive days for a total of 2 hr. Each medium was inoculated with 5 ml of pycnidiospore suspension, and flasks were maintained under conditions similar to those described for production of pycnidiospores. Pycnidiospores were harvested 28 days after inoculation of media. Germination was evaluated by placing two drops of pycnidiospore suspension on glass slides coated with a film of Gelgard and incubating the slides in a moist chamber (100% relative humidity) for 18–24 hr. A total of 100 spores was counted, and the percentage of those with germ tubes twice the diameter were considered to have germinated.

RESULTS

Sporulation of *S. macrospora* and *S. maydis* in basal mineral salts was influenced by the concentration of both sucrose and biotin. With sucrose, the maximum *S. macrospora* sporulation of 0.31×10^6 pycnidiospores per milliliter was obtained with 10 g/L and the maximum *S. maydis* sporulation of 3.3×10^6 pycnidiospores per milliliter was obtained with 5 g/L (Table 1). There was a negative correlation ($r = -0.98$ and -0.50 for *S. maydis* and *S. macrospora*, respectively) between sucrose concentration and sporulation. Dry weight of mycelia produced by each organism, on the other hand, increased with increasing concentrations of sucrose (Table 1). There was, therefore, a positive correlation ($r = 0.65$ and 0.98 for *S. maydis* and *S. macrospora*, respectively) between vegetative yield and sucrose concentration. For both organisms, there was an inverse relationship between sporulation and yield of mycelia.

With 6 mg/L of biotin, *S. maydis* produced 2.3×10^6 pycnidiospores, which was significantly higher than with any other concentration. Pycnidiospore production by *S. macrospora* at 6 and 10 mg/L of biotin was similar, and production was not significantly greater at higher concentrations (Table 2). Biotin concentration higher or lower than 6 mg/L depressed sporulation of *S. maydis*. Sporulation of *S. macrospora* was greater with biotin in the medium, indicating that it is required by this species. Production of mycelia by each organism was greater in the presence of biotin. The least amount of mycelia produced by *S. macrospora* was obtained with 0 mg/L of biotin (Table 2).

Pycnidiospore germination of *S. maydis* was 86 and 96% on oat and basal mineral salts media, respectively, and that of *S. macropsora* was 85 and 96%, respectively.

DISCUSSION

A basal mineral salts medium amended with sucrose (10 g/L) and biotin (6 mg/L) consistently yielded at least 2×10^6 and 1×10^6 pycnidiospores per liter for *S. maydis* and *S. macrospora*, respectively. Pycnidiospores were morphologically similar to published descriptions of the organisms (1–3,11). Consistent results were obtained, however, only if glass-distilled deionized water was used. Apparently, pyrogens and other substances in water treated by other methods inhibit sporulation. Also, cultures should not be disturbed when growth of mycelia from the plugs begins, since agitation apparently inhibits sporulation.

Although *S. maydis* and *S. macrospora* have been grown on organic substrates (1,7), few quantitative studies of growth, sporulation, and germination requirements have been reported. These deficiencies may be due almost exclusively to the lack of culture media that yields morphologically similar pycnidiospores. Methods that employ colonization of organic substrates (sorghum, oats, or wheat kernels) for pycnidiospore production are not suitable for accumulation of quantitative data. Studies on artificial media have been limited, in part, by slow growth and poor sporulation. Our studies show that pycnidiospores produced on the amended basal mineral salts medium are morphologically uniform.

One advantage of using this medium for production of pycnidiospores is that agitation of cultures is not needed, as when pycnidiospores are produced on organic media, a more time-consuming procedure. Also, by varying the concentration of sucrose and biotin in the medium, large quantities of mycelia can be produced with the same ease as pycnidiospores are produced. Pycnidiospores can be harvested by homogenizing flask contents in a commercial blender, then comminuting the suspension through cheesecloth. We have inoculated corn with unfiltered homogenate by injecting the stalk with a syringe or by spraying into the leaf whorl with a hand garden sprayer. Neither piece of equipment was clogged.

With this growth medium, less time and space are required to produce large numbers of pycnidiospores. Because less time is required and pycnidiospore concentration is higher, less room is required for maintaining containers used in production. The growth medium described should be adopted to reduce errors in morphologically and physiologically

Table 1. Effect of sucrose in basal mineral salts medium on sporulation and mycelia production by *Stenocarpella maydis* and *S. macrospora* after 28 days at 23 C[y]

Sucrose concentration (g/L)	Sporulation (pycnidiospores × 10^6/ml)		Mycelia dry weight (mg)	
	S. maydis	*S. macrospora*	*S. maydis*	*S. macrospora*
0	0.000 a[z]	0.000 a	4.4 a	16.1 a
5	3.300 b	0.014 a	78.1 b	15.5 a
10	3.000 b	0.308 c	131.4 c	130.1 b
15	0.820 a	0.158 b	185.0 d	183.5 c
20	0.150 a	0.088 ab	232.8 e	213.1 cd
25	0.009 a	0.023 a	231.7 e	244.2 de
30	0.003 a	0.003 a	280.5 f	270.2 e

[y] Average of three tests and three replications each.
[z] Treatment means with different letters are significantly different according to Duncan's multiple range test ($P = 0.05$).

Table 2. Effect of biotin in basal mineral salts medium containing 10 g/L of sucrose on sporulation and mycelia production by *Stenocarpella maydis* and *S. macrospora* after 28 days at 23 C[y]

Biotin concentration (mg/L)	Sporulation (pycnidiospores × 10^6/ml)		Mycelia dry weight (mg)	
	S. maydis	*S. macrospora*	*S. maydis*	*S. macrospora*
0	0.733 a[z]	0.000 a	3,583 a	1,466 a
2	1.310 b	0.038 b	3,518 a	3,728 b
4	1.140 b	0.058 c	3,546 a	3,338 b
6	2.300 d	0.087 d	3,026 a	2,978 b
8	1.480 c	0.089 d	3,069 a	3,259 b
10	1.760 e	0.080 d	3,222 a	3,495 b
20	0.081 a	0.062 c	3,379 a	3,489 b
30	1.710 e	0.055 cd	3,286 a	3,575 b
40	1.200 b	0.045 e	3,347 a	3,501 b
50	0.810 a	0.078 d	3,258 a	2,894 b

[y] Average of three tests and three replications each.
[z] Treatment means with different letters are significantly different according to Duncan's multiple range test ($P = 0.05$).

characterizing these two species. Murphy et al (7) noted morphological differences in formation of *S. maydis* pycnidia grown in synthetic media vs. host tissue, and they concluded this was due to differences in the nutritional environment. In our opinion, if the nutrient source is readily available and complex substances do not need to be broken down, then pycnidiospores should be more viable than ones having to acquire exogenous nutrients. Maintenance of the organisms on a nonhost could only add further to the inaccuracy in the characterization of *Stenocarpella* species.

We have successfully and repeatedly used this medium and methodology to produce large numbers of pycnidiospores for use in studies evaluating the effect of these pathogens on maize. It is our recommendation, therefore, that researchers evaluating diseases incited by *Stenocarpella* species use this growth medium to ensure more consistency and uniformity of results.

ACKNOWLEDGMENTS

We thank G. C. Bergstrom and E. M. Bauske for their advice, critical reading of the manuscript, and valuable suggestions.

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