Sporulation and Growth of *Curvularia pallescens* as Affected by Media, Temperature, and Nitrogen Sources

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ABSTRACT

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Sporulation on mycelial disks of *Curvularia pallescens* plated on agar media was optimum at 15 C, and water agar was a better medium than various nutrient agars. Asparagine and DL-aspartic acid were the best organic nitrogen sources for sporulation, and inorganic nitrogen sources were less favorable in liquid media. This same temperature was also optimum for sporulation on leaf tissue of the maize host. On the contrary, sporulation on plates directly seeded with spores was most abundant at 24 C, and malt extract agar and potato-dextrose agars were superior to water agar. Viability of the spores decreased with increase in culture age, and after 10 mo at 10 C the spores had totally lost viability. For growth in defined liquid culture, the order of preference of inorganic nitrogen sources was nitrate, ammonium, and nitrite. Glycine was utilized significantly better than any of the other organic or inorganic nitrogen sources tested. However, asparagine, arginine, and glutamic acid supported good mycelial growth while urea, tryptophane, and L-leucine were poor in this regard.

Additional key words: *Zea mays*.

The symptoms of the leaf spot caused in maize by *Curvularia pallescens* have been studied extensively (7). Further work on *C. pallescens* concentrated on control measures with zineb fungicide on maize farms in Nigeria (4).

Studies on nutrition and growth of the pathogen would be useful in future investigations on the physiology of the fungus and on the possible production of toxins or enzymes involved in pathogenesis (8). Also, knowledge of cultural methods and conditions to promote spore production would be advantageous in obtaining adequate amounts of inoculum.

These studies were conducted to determine the optimum temperature, appropriate substrate, method of cultivation, and types of nitrogen sources for growth and sporulation of *C. pallescens*.

MATERIALS AND METHODS

Curvularia pallescens was isolated from diseased maize leaves on the experimental site of the National Cereals Research Institute, Moor Plantation, Ibadan, Nigeria. Pathogenicity was confirmed by tests on healthy maize plants in the screenhouse. Stock cultures were maintained on potato-dextrose agar (PDA) and water agar (WA).

The solid media used were: PDA, WA, cornmeal agar (CMA), malt extract agar (MEA), nutrient agar (NA), yeast extract agar (YEA), dextrose agar (DA), and potato agar (PA). These media were prepared in accordance with manufacturer's recommendations. They were sterilized by autoclaving at 1.1 kg/cm^2 (121 C) for 15 min, and 15 ml was dispensed into sterile petri dishes.

The basal liquid medium used for nitrogen nutrition studies was the synthetic culture medium described by Kurtz and Fergus (6) and had the following composition: $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 1.0 g; thiamin, 0.1 mg; biotin, 0.005 mg; Fe, 0.2 mg; Zn, 0.2 mg; and Mn, 0.2 mg in 1 L of distilled water. The nitrogen source was added in a quantity to yield an amount of nitrogen equal to that in 2 g of asparagine. For organic nitrogen sources, the carbon equivalent of the compound was calculated, and then a quantity of glucose was added to the basal medium to yield a total of 4 g of carbon per liter (glucose carbon plus the amount of carbon added in the organic nitrogen compound).

All components of medium except the nitrogen sources were combined at twice the desired final concentration, dispensed in 25-ml portions in 250-ml Erlenmeyer flasks, and sterilized as previously indicated. Twenty-five milliliters of the nitrogen solution at twice the desired final concentration was sterilized alone in test tubes, cooled, and aseptically poured into a sterile Erlenmeyer flask. Urea was sterilized by seitz filtration. The solutions were adjusted to the required pH by adding sterile 0.1 N HCl or 0.1 N NaOH.

Mycelial inoculations of media were made using 6-mm-diameter disks cut from the margins of 5-day-old PDA cultures of the fungus with a sterile cork borer. They were placed at the center of the plates of solid media or simply dropped into the liquid media. Conidial inoculations of petri plates containing solid media were initiated by adding 0.1 ml of spore suspension containing 5,000 spores per milliliter of distilled water. Sporulation by cultures grown from spores was enhanced by inoculating them in a broadcast pattern. The spores used for inoculating media were collected from test tube slants and were not washed before transfer.

Both the mycelial and conidial inoculation experiments were incubated for 7 days at 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 C. In the nitrogen studies, growth of shaken and stationary cultures as well as sporulation were compared at 24 C. The shaken cultures were placed on an orbital shaker at 80 rpm. The treatments were replicated four times, and a nitrogen-free control flask was included with each one.

Spores were harvested from the solid media by pouring 10 ml of distilled water into the petri dishes, allowing it to stand for 1 min, and using a camel's-hair brush to dislodge the spores. The contents of the dishes were shaken and sieved through two layers of muslin cloth to remove pieces of agar and mycelia. A hemocytometer was used to count the spores. Also, the contents of the liquid cultures were carefully removed from the flask and placed inside a clean, sterile petri dish containing 10 ml of distilled water. Spores were extracted and counted as in the solid media experiments. Afterwards, the mycelium was collected on a filter paper that had been previously dried in a hot air oven at 80 C and weighed to the nearest milligram. The mycelium was rinsed three times with 5 ml of sterile distilled water, dried overnight at 80 C, and weighed. The filtrates from the four replicates were collected and their pHs determined with a Beckman pH meter.

To test the effect of age on viability of spores, sporulating WA and MEA cultures were placed in storage at 10 C, and each month for 10 mo, samples of spores were removed and tested for

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germinability. The spores were washed and placed on microscope slides in a chamber at 25 C and ~100% RH.

To study sporulation on infected leaf tissues, maize seedlings were raised in the greenhouse in medium-sized plastic pots for 21 days. They were sprayed to runoff with a spore suspension of *C. pallescens*. After 1 wk, leaf lesions were excised and washed under running tap water for about 1 hr to remove spores that might have formed during lesion development. The excised lesion areas were incubated for 7 days under the same temperatures used in previous experiments and examined microscopically for sporulation.

RESULTS

Influence of temperature on sporulation. Temperatures of 15, 12, and 18 C in that order supported increased production of large numbers of spores when mycelial plantings on WA were used (Fig. 1). Sporulation at 12 and 15 C was not significantly different but both were significantly higher than at 18 C and other temperatures. No sporulation occurred in cultures maintained at 3 and 6 C and very little occurred at 30 C. These temperature effects were similar on all the media used.

When cultures were initiated by sowing spores, sporulation increased greatly from 9 to 24 C, after which there was a decrease to 30 C (Fig. 2). No sporulation was observed at 3 and 6 C. The greatest numbers of spores were produced at 21 and 24 C with 2,600 and 2,700 spores per milliliter of distilled water, respectively. Numbers of spores produced at 21 and 24 C were not significantly different, but both were significantly different from those produced at other temperatures.

Microscopic observation of the washed and incubated infected maize leaf tissue showed abundant sporulation at 12, 15, and 18 C. Sporulation did not occur at 3 and 6 C, but a few spores formed at 9, 21, 24, 27, and 30 C.

Influence of medium on sporulation. When agar media were seeded with mycelial disks and incubated at 15 C for 7 days, *C. pallescens* produced about twice as many spores on WA as on MEA, PDA, or PA (Fig. 3). The proportions of spores produced on YEA, CDA, CMA, and NA, as compared with WA, were ~0.4, 0.25, 0.2, and 0.16, respectively. The quantity of mycelium produced on different agar media was not determined, but it appeared that cultures on nutrient media produced more mycelium than those on WA.

Spore production on media inoculated with spores of *C. pallescens* and incubated at 24 C differed from that on media inoculated with mycelial disks and incubated at 15 C (Fig. 4). The suitability of media was rated in the following decreasing order: MEA, PDA, PA, CMA, NA, YEA, CDA, and WA.

In liquid cultures with various nitrogen sources, DL-aspartic acid promoted the best sporulation of all the nitrogen sources used (Table 1). This was closely followed by asparagine, DL-isoleucine, L-glutamic acid, tyrosine, and tryptophane in that order. As a group, organic nitrogen sources supported sporulation better than the inorganic nitrogen sources.

Effect of age of culture on spore viability. In sporulating WA slant cultures stored at 10 C up to 10 mo, percentage germination decreased significantly from the second until the fifth month (Fig. 5). After the fifth month, low germination was obtained with no significant difference until the ninth month, after which germination ceased.

When sporulating MEA cultures were stored at 10 C, percentage germination of the spores was higher in the first month with a nonsignificant decrease in the second month (Fig. 6). After the second month, percentage germination of the spores fell to about 42% in the third month and decreased further in subsequent months till the sixth month. There was relatively low germination

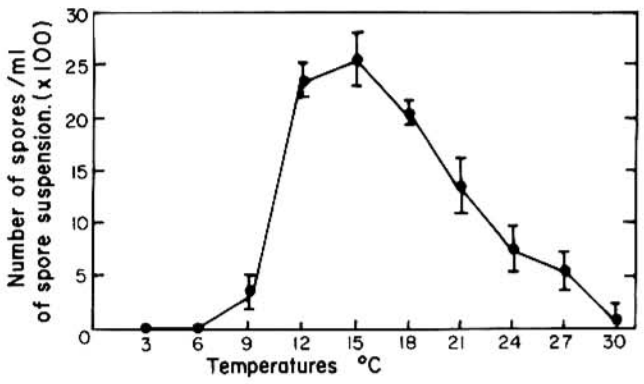

Fig. 1. Sporulation of *Curvularia pallescens* on mycelial disks plated on water agar and incubated at different temperatures for 7 days.

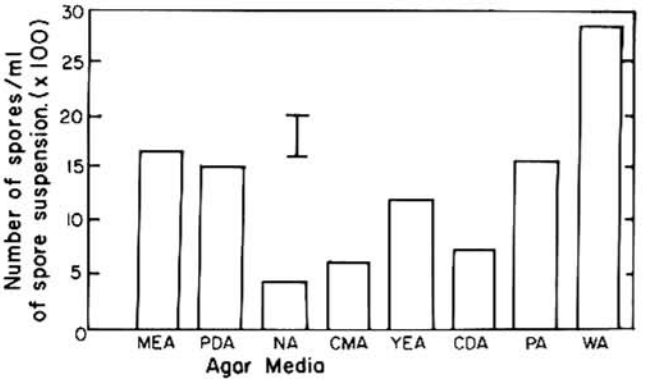

Fig. 3. Sporulation on mycelial disks of *Curvularia pallescens* plated on different agar media and incubated at 15 C for 7 days. l = least significant difference ($P = 0.05$).

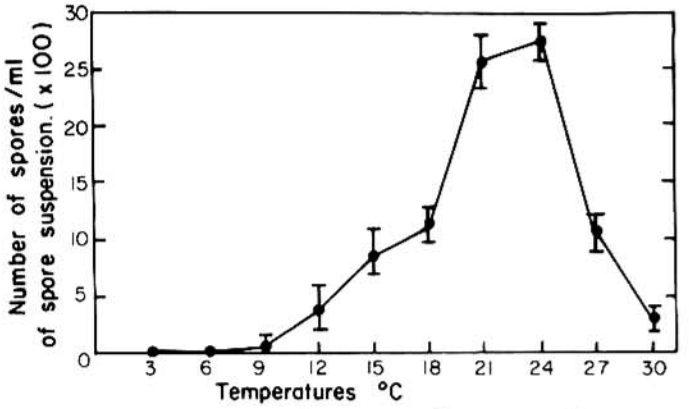

Fig. 2. Sporulation of colonies of *Curvularia pallescens* grown from spores sown on malt extract agar and incubated at different temperatures for 7 days.

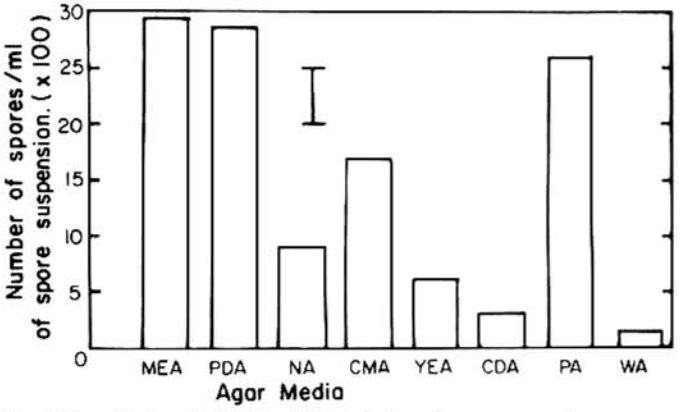

Fig. 4. Sporulation of colonies of *Curvularia pallescens* grown from spores sown on different agar media and incubated at 24 C for 7 days. l = least significant difference ($P = 0.05$).

of spores from 7- to 9-mo-old cultures. No germination was obtained at 10 mo.

Growth on different nitrogen sources. Shaken and stationary cultures did not give significantly different results during the course of the experiment; thus, only the data for stationary cultures are presented. Growth was best in organic nitrogen sources, with glycine significantly leading all others (Table 1). Furthermore, $NaNO_3$ led the inorganic nitrogen group, but its capability to support growth was significantly lower than that of glycine. Of all the inorganic nitrogen sources used, $NaNO_2$ and NH_4Cl supported the least growth (Table 1). However, urea supported the least growth when all nitrogen sources were compared. Autolysis and loss in mycelial dry weight started earlier in $NaNO_3$ than in glycine (Fig. 7). At the termination of the experiment, the glycine cultures still had the greatest amount of growth. However, *C. pallescens* grew well in the organic and inorganic nitrogen sources.

The pH values did not follow the same trend in the different nitrogen sources. The pH of the glycine medium rose from 4.5 to 7.7, 30 days after growth, while that on $NaNO_3$ rose from 6.2 at 10 days to 6.5 on the 20th day and finally dropped to 6.4 on the 30th day of growth (Table 1). The control flask had scanty growth and the pH did not change very much during the experiment.

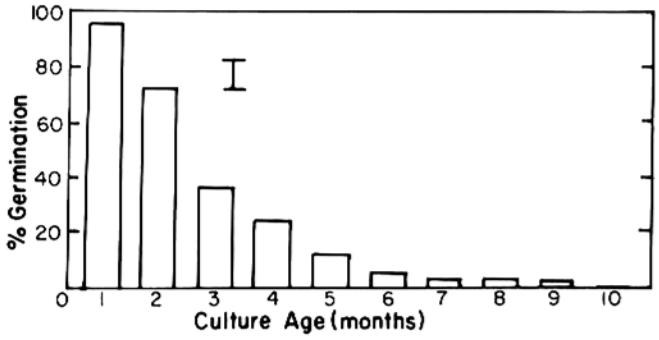

Fig. 5. Germination of spores harvested at monthly intervals from cultures of *Curvularia pallescens* stored on water agar at 10 C. I = least significant difference ($P = 0.05$).

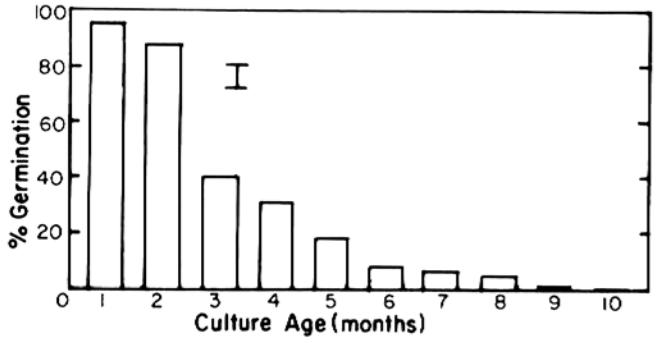

Fig. 6. Germination of spores harvested on monthly intervals from cultures of *Curvularia pallescens* stored on malt extract agar at 10 C. I = least significant difference ($P = 0.05$).

DISCUSSION

Production of spores of *C. pallescens* in culture could be carried out in two ways: by initiating culture from mycelium, or from spores. Furthermore, different sets of conditions supported the best sporulation obtained by the two methods. Chang and Tyler (1) noted similar results with *Cercosporella herpotrichoides* Fron.

Temperature played a major role in sporulation of the fungus. The optimum temperature for sporulation varied according to the method of inoculation of the fungus. Moderately high temperature was distinctly more favorable when cultures were started from spores (on MEA) than when started from mycelium (on WA). This seemed to be in conformance with the findings of Chi and Hanson (2) in tests of different temperature regimes on the sporulation of *Fusarium* spp. It could be that the spores, more than the mycelium, needed higher temperature for stimulating enzymatic activity, germination, and germ tube development prior to sporulation (5,9).

Nutrient effect on sporulation also varied between the two inoculation methods. Scanty nutrient promoted sporulation when mycelium was used for inoculum, while nutrient-rich substrate promoted direct sporulation when spores were plated. At 24 C, MEA or PDA seeded with spores was about 20 times more suitable than WA for supporting sporulation. Chi and Hanson (2) obtained a similar result with *Fusarium* spp. The mycelial inoculum needed less nutrient, whereas spores required more nutrient for

TABLE 1. Dry weights of mycelium and spore production by *Curvularia pallescens* and the pH of the liquid culture media of different nitrogen sources

	Incubation								
	10 days			20 days			30 days		
Nitrogen sources	Mycelium[y] (mg)	Spores[z] (no.)	pH	Mycelium (mg)	Spores (no.)	pH	Mycelium (mg)	Spores (no.)	pH
DL-Aspartic acid	120 g	370 a	6.8	294 b	1,550 a	7.8	120 d	1,600 a	8.3
Asparagine	260 b	160 bc	6.3	310 b	810 b	7.3	288 b	1,028 b	7.4
Glycine	300 a	90 cd	4.5	460 a	146 f	6.9	345 a	494 d	7.7
DL-Alanine	210 d	70 cd	6.6	167 e	200 e	7.9	143 f	307 e	7.6
L-Arginine	240 c	160 bc	4.6	281 bc	500 bc	7.4	213 d	624 c	7.3
L-Glutamic acid	283 b	150 bc	7.6	280 c	600 bc	6.9	244 c	771 c	6.9
DL-Isoleucine	200 d	140 bc	6.2	280 bc	724 b	7.8	214 d	834 c	7.3
L-Phenylalanine	160 e	50 d	4.0	190 de	240 de	7.7	146 f	420 d	7.7
L-Methionine	214 d	73 cd	5.1	200 d	168 e	8.1	171 e	360 e	6.9
L-Leucine	114 g	180 b	3.8	110 f	416 c	7.4	80 g	592 d	7.9
DL-Valine	164 e	80 cd	4.8	190 de	193 e	7.7	184 de	343 e	8.1
DL-Threonine	143 f	120 c	4.4	180 c	200 e	6.9	154 fg	584 cd	8.3
DL-Histidine	124 g	210 b	4.6	111 f	400 c	7.4	101 fg	584 cd	7.6
L-Typtophane	148 ef	163 bc	5.9	92 g	643 bc	7.9	100 fg	710 c	4.9
Urea	106 h	194 b	7.8	98 g	593 bc	8.0	83 g	621 c	7.9
NH_4NO_3	191 h	151 bc	6.8	92 g	310 d	5.1	81 g	464 d	6.0
$NaNO_3$	175 e	120 c	6.1	300 b	424 c	6.4	205 d	595 cd	6.2
$NaNO_2$	75 i	90 cd	6.2	118 f	180 e	6.5	103 fg	310 e	6.4
$(NH_4)_2SO_4$	157 e	110 c	4.5	210 d	364 d	4.9	121 f	380 e	4.3
NH_4Cl	73 i	90 cd	4.8	120 f	296 d	4.9	118 f	410 d	4.7
Control	8 j	180 b	6.2	21 h	224 e	6.4	29 h	310 e	6.3

[y] Mean values (in the same column) not followed by the same letter are significantly different, $P = 0.05$, according to Duncan's multiple range test.
[z] Spores per milliliter of spore suspension. Data are means of four replicates.

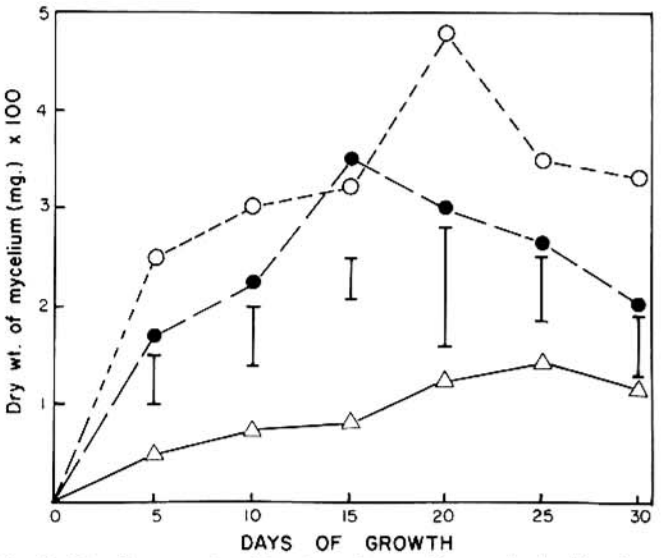

Fig. 7. Mycelium produced by *Curvularia pallescens* in liquid culture containing glycine (circles), $NaNO_3$ (filled circles), and NH_4Cl (triangles) as sources of nitrogen. l = least significant difference ($P = 0.05$).

development before sporulation. As far as nitrogen nutrition is concerned, DL-aspartic acid and asparagine supported more sporulation than the inorganic nitrogen sources. Also, nitrate nitrogen was better than nitrite or ammonium.

Sporulation on incubated infected maize leaf pieces virtually reproduced the mycelium planting method, in that the washed leaf tissue contained embedded mycelium without spores. In conformance with this, the temperature conditions that favored sporulation by the mycelium-planting method also favored that of the washed infected leaf tissue.

The inability of 10-mo-old spores to germinate indicated loss of viability (1). This occurred both on a nutrient-poor medium (WA) and on a nutrient-rich medium (MEA).

The capability of *C. pallescens* to grow well in a wide range of organic and inorganic nitrogen sources gives it the opportunity to survive on various substrates with different nitrogen sources. This is also true of most fungi (3,6). The optimum nitrogen source (glycine) for *C. pallescens* is one of the best sources for most plant parasitic fungi (3). Also, as has been found for many fungi (3), urea, tryptophane, and L-leucine supported poor growth. $NaNO_3$ was the best of the inorganic nitrogen sources. Other fungi, such as *Colletotrichum coccodes* (6), also utilize nitrate nitrogen well.

In most media, autolysis commenced between 15 and 20 days of growth, while the pH decreased at first and then increased beyond the initial pH of 6.0. Ammonium nitrogen sources as well as the less suitable organic nitrogen sources might support better growth in a well buffered medium. It appears that $(NH_4)_2SO_4$ would probably be the best inorganic nitrogen source if the pH could be maintained at about 8. This was indicated in the experiments of Thind and Rawla (10). However, there is doubt about the occurrence of such a high pH in living tissues.

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