# Influence of Nutrition During Conidiation of *Colletotrichum truncatum* on Conidial Germination and Efficacy in Inciting Disease in *Sesbania exaltata*

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## ABSTRACT

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Conidia of *Colletotrichum truncatum* (NRRL 13737) were produced in semidefined, liquid media, with total carbon concentrations of 4 g/L and carbon/nitrogen ratios of 40:1, 15:1, and 5:1. Conidia produced in 5:1 medium were longer and thinner than conidia from 15:1 and 40:1 media, and a higher proportion contained two, rather than one, nuclei per conidium. After either 6 or 12 hr on cellophane membranes, a greater proportion of conidia produced in the 5:1 medium had germinated compared with conidia from 15:1 and 40:1 media. Germination on attached leaves of *Sesbania exaltata* was greatest with conidia from 5:1 medium when assayed after either 6 or 24 hr. Equality of variance tests implied that the leaf environment had a greater influence on the germination of conidia from 15:1 medium than conidia from 40:1 or 5:1 media. All conidial treatments caused losses in biomass of seedlings of *S. exaltata*. Conidia produced from 5:1 or 15:1 media induced greater reduction in shoot height, and conidia from 5:1 medium induced greater reduction in shoot dry weight than did conidia from 40:1 medium. Further research on increasing the efficacy of mycoherbicide conidia by modifying the phyllosphere environment and the nutritional conditions of the conidiation medium is needed.

*Additional keywords*: bioherbicide, hemp sesbania, nuclear number, phylloplane, sporulation, weed.

Hemp sesbania (*Sesbania exaltata* (Raf.) Rydb. ex A. W. Hill) is a weed of increasing importance in soybean, cotton, and rice crops, especially throughout the Coastal Plain and Piedmont regions of the southeastern United States (5). *Colletotrichum truncatum* (Schwein.) Andrus and W. D. Moore has potential as a mycoherbicide against *S. exaltata* (4).

Interest in using host-specific plant pathogens to control weedy plants has increased as chemical herbicide registrations have declined and public interest in agricultural products produced with fewer pesticides has risen. To manufacture an economically feasible mycoherbicide product, large-scale liquid fermentation techniques were developed for the commercial production of conidia of *Colletotrichum gloeosporioides* f. sp. *aeschynomene* (Collego; 6). The cost-effective production of fungal spores from potential bioherbicide agents is essential in the commercialization of these products (3). Nutritional environments that increase the production of spores of other potential mycoherbicide agents in liquid culture (M. A. Jackson, *unpublished results*; 16), on sodium alginate granules (29), or on the surface of mycelial slurries (27) have been reported. Whether nutritional conditions that maximize sporulation optimize the potential of the spores to germinate and incite disease in a susceptible weed host has received little attention.

Solid substrates of various nutritional compositions influenced conidial size, nuclear number, germination, and virulence of a variety of fungal pathogens (15,20,21). The influence that the nutritional environment has on these conidial traits when conidia of pathogenic fungi are produced in liquid culture is uncertain.

*C. truncatum* was selected for use in this investigation because of its considerable potential as a mycoherbicide. Sporulation of *C. truncatum* in liquid culture is influenced significantly by the conidiation medium (9). Our objective was to determine if producing conidia of *C. truncatum* in liquid media with differing carbon/nitrogen (C/N) ratios would differentially influence conidial size, nuclear number, germination, and efficacy in inciting disease in *S. exaltata*.

## MATERIALS AND METHODS

**Production and harvest of conidia.** An isolate of *C. truncatum* (NRRL 13737, = ARS patent culture collection #18434), which originally was obtained from diseased seedlings of hemp sesbania, was used in all studies. The fungus was stored at $-80$ C in 10% glycerol on colonized 2-mm-diameter potato-dextrose agar (PDA; Difco Laboratories, Detroit, MI) plugs until needed.

The basal salts portion of the liquid medium (BSM) used to produce conidia consisted of: 2.0 g of $KH_2PO_4$; 0.4 g of $CaCl_2 \cdot 2H_2O$; 0.3 g of $MgSO_4 \cdot 7H_2O$; 50 mg of $FeSO_4 \cdot 7H_2O$; 37 mg of $CoCl_2 \cdot 6H_2O$; 16 mg of $MnSO_4 \cdot 7H_2O$; 14 mg of $ZnSO_4 \cdot 7H_2O$; 500 $\mu g$ each of thiamine, riboflavin, pantothenate, niacin, pyridoxamine, and thioctic acid; and 50 $\mu g$ each of folic acid, biotin, and vitamin $B_{12}$ per liter of deionized water. A stock solution of sterile, autoclaved glucose (20%, w/v; Difco) was added to cooled, autoclaved (121 C for 15 min) BSM, whereas casamino acids (Difco) were added to BSM before autoclaving. Media with final C/N ratios of approximately 40:1, 15:1, and 5:1 were obtained by adding glucose/casamino acid at 9.2 g/0.6 g, 7.8 g/1.7 g, and 3.5 g/4.9 g/L, respectively; each medium had a total of 4 g/L of carbon but varied in the proportion of carbon supplied by glucose. Calculations of C/N ratios were based only on the amount of carbon and nitrogen present in glucose and casamino acids. The final pH of all media was adjusted to 5.0 with 2N NaOH or 2N HCl before autoclaving, and each medium was dispensed in three 1,000-ml flasks (500 ml/flask) having baffles (Bellco, Inc., Vineland, NJ).

Conidial suspension for the initiation of liquid cultures was produced by transferring PDA plugs taken from $-80$ C glycerol cultures to petri dishes of PDA and incubating at $25 \pm 3$ C with daily 12 hr periods of ambient light for 7-10 days on the

laboratory bench. Conidia then were harvested from dishes with sterile-deionized water, and the concentration of the resultant conidial suspension was adjusted to $5 \times 10^5$ conidia per milliliter with the aid of a hemacytometer. Sufficient conidial suspension was added to each flask to obtain a final conidial concentration of $5 \times 10^4$ conidia per milliliter. Flasks were closed with loose-fitting stainless steel caps and incubated in the dark for 6 days in an environmentally controlled orbital shaker (300 rpm and $28 \pm 1$ C; Environ-Shaker, Labline Instruments, Inc., Melrose Park, IL). During incubation, flasks were shaken by hand daily to minimize fungal growth on the flask walls, and the pH of each medium was checked daily and maintained at pH 5.0 with the addition of either 2N NaOH or 2N HCl as needed.

Conidia were harvested after 6 days, and the contents of the three flasks of each treatment were combined to produce populations of spores for subsequent experimental evaluation. Spore concentrations of approximately $6.5 \times 10^6$, $1.9 \times 10^7$, and $7.6 \times 10^6$ per milliliter were obtained in the 40:1, 15:1, and 5:1 media, respectively, at harvest. Conidial numbers neared maximal concentrations in each medium at this time and reached maximal concentrations of approximately $7.0 \times 10^6$, $2.1 \times 10^7$, and $7.8 \times 10^6$ per milliliter, respectively, after incubation for 7 days (9).

Conidial suspensions were filtered through six layers of sterile gauze, centrifuged (7 min at $7,000 \times g$ maximum, 4 C; GSA rotor, Sorvall Instruments, Wilmington, DE), and conidial pellets were resuspended in sterile water. After a second centrifugation, $2 \mu l$ of conidial pellets from each treatment was set aside for determination of conidial size and nuclear number. Conidia then were resuspended in an aqueous solution of 0.04% (v/v) wetting agent (Triton X-100; Sigma Chemicals, St. Louis, MO), and conidial suspensions of three different concentrations were prepared for use in germination and inoculum efficacy studies.

**Size and nuclear number of conidia.** Washed conidial pellet samples from each treatment were resuspended in 5 ml of Carnoy's fluid (75:25, v/v, of absolute ethanol and glacial acetic acid, respectively). Drops of conidial suspensions were placed on glass slides coated with 0.1% carrageenan and, after 24 hr, stained with Giemsa-HCl dye (28). Coverslips were affixed with Permount adhesive (Fisher Scientific, Pittsburgh, PA). Nuclear number was determined by examining 100 conidia from each of four samples per treatment, whereas 20 conidia per sample were measured for length and width. Nuclear number data (percentages) were transformed to arcsine-square roots and subjected to analysis of variance (ANOVA).

**Germination of conidia on cellophane membranes.** Petri dishes containing 2% water agar were overlaid with cellophane membrane backing (Bio-Rad Laboratories, Richmond, CA), which had been cut to fit the 9-cm-diameter dishes and sterilized by autoclaving for 15 min in deionized water. Conidial suspensions in 0.04% wetting agent ($1 \times 10^6$ conidia per milliliter) were misted onto membranes until wetted. Dishes were stored in the dark at $24 \pm 1$ C in a completely randomized design. After 3, 6, and 12 hr, Carnoy's fluid was sprayed onto the surface of four plates per treatment to arrest the germination of spores on membranes. Four dishes from each treatment were examined microscopically for each assay time, and 100 conidia per plate evaluated for germination or germination and appressorial formation. Because the normality of the data was satisfactory, data were analyzed without arcsine-square root transformation by one-way ANOVA.

**Germination of conidia on attached leaves of S. exaltata.** Seeds of *S. exaltata* were sown in 7.5-cm-tall (100 cm$^3$) peat pots containing potting mix (Terra-lite Rediearth; W. R. Grace, Cambridge, MA). Seedlings were thinned to one per pot after emergence, and were grown in a greenhouse at $23 \pm 4$ C under ambient light supplemented with sodium vapor lamps (average total photon flux density was 300 $\mu$mol m$^{-2}$s$^{-1}$). Two weeks after seeding, plants were fertilized with 5 ml/pot of a stock solution of Peters 20-20-20 (1.8 g/L; W. R. Grace, Fogelsville, PA) and transferred to a growth cabinet. Seedlings were grown for an additional week at $26 \pm 1$ C, 60–80% relative humidity, and with 14 hr of fluorescent and incandescent light (290 $\mu$mol m$^{-2}$s$^{-1}$). Conidial suspensions in 0.04% wetting agent ($2 \times 10^6$ conidia

per milliliter) were sprayed onto eight seedlings per treatment until runoff. Inoculated seedlings were immediately placed in a completely randomized design in a dew chamber without lights at $26 \pm 1$ C. After 6 and 24 hr, the oldest compound leaf from each of four seedlings per treatment was removed, and the second and fourth pair of leaflets mounted on glass slides with double-sided transparent tape. Carnoy's fluid was briefly sprayed onto leaflets; the leaflets were stained with a drop of 0.01% acridine orange (Sigma) in deionized H$_2$O; and 50 conidia per leaflet were examined for germination and appressorial formation with epifluorescent microscopy. Seedling, leaflet position, and leaflet pair variables did not influence germination or appressorial formation, as determined by initial analysis. Thus, these variables were not included as sources of variation in a one-way ANOVA. Sample variances for germination and appressorial formation data sets were compared with Fisher's equality of variance test (23).

**Efficacy of conidia in inciting disease symptoms in S. exaltata.** Plants were produced as described above. After the 3-wk growth period, seedlings were blocked by size into four groups of 20 seedlings each before inoculation. Solutions of 5:1, 15:1, or 40:1 treatments ($5.5 \times 10^5$ conidia per milliliter) were sprayed until runoff onto a total of 20 seedlings for each conidial treatment. Each treatment group of 20 seedlings was composed of five seedlings from each of the four size groupings. A solution of 0.04% wetting agent was sprayed onto 20 control seedlings. Plants were held for 23 hr at $26 \pm 1$ C in a dew chamber and then placed in a randomized complete block design in a growth cabinet under the environmental conditions described earlier. Plants were watered with deionized water as needed and harvested after 8 days. Plants were scored for top height, shoot dry weight, and the number of leaves per seedling.

Data were subjected to a two-way ANOVA. This and all other experiments were repeated at least twice, and mean separations were performed with Fisher's protected LSD at $P = 0.01$. Data presented are from a single experiment and are representative of results obtained from repeated experiments.

## RESULTS

**Spore size, nuclear number.** Conidia produced in 5:1 medium were longer and thinner than conidia from 15:1 and 40:1 media, whereas conidia from 15:1 medium were intermediate in length and width (Table 1). In comparison with conidia from the other treatments, a higher proportion of conidia from 5:1 medium contained two, rather than one, nuclei per conidium (Table 1). Conidia produced in 15:1 medium most consistently contained one nucleus per conidium.

**Germination of conidia on cellophane membranes.** After either 3, 6, or 12 hr on cellophane membranes, a greater proportion of conidia from 5:1 medium had germinated than had conidia from the other treatments (Table 2). Conidia from 15:1 medium had germinated to the least extent at all assay times. After 6 or 12 hr, a greater proportion of conidia from 5:1 medium had germinated and formed appressoria than had conidia from the other media. Forty-six percent of conidia from 40:1 and 18%

TABLE 1. Average dimensions ($\mu$m) and nuclear number frequency (%) of conidia of *Colletotrichum truncatum* produced in liquid media of differing nutritional environments

| Carbon/nitrogen ratio of conidiation medium | Conidial length | Conidial width | Nuclear number frequency (%) | | |
|---|---|---|---|---|---|
| | | | 0[y] | 1 | 2 |
| 40:1 | 14.9 c[z] | 2.7 a | 4.0 a | 95.5 b | 0.5 b |
| 15:1 | 16.0 b | 2.1 b | 1.2 a | 98.8 a | 0.0 b |
| 5:1 | 17.7 a | 2.0 c | 1.2 a | 95.5 b | 3.2 a |
| LSD | 0.6 | 0.1 | ... | 3.0 | 1.6 |

[y] Number of nuclei per conidium.

[z] Values within columns followed by the same letter are not significantly different ($P \leq 0.01$). Values represent means of samples taken from a population of spores produced in the conidiation media shown.

of conidia from 15:1 media had germinated after 24 hr. Extensive hyphal growth on membranes precluded collecting 24-hr data for conidia from the 5:1 medium.

**Germination of conidia on attached leaves of *S. exaltata*.** A greater proportion of conidia from 5:1 medium germinated on leaves of *S. exaltata* after both 6 and 24 hr than did conidia from 15:1 and 40:1 media (Table 3). At both assay times, a higher proportion of germinated conidia from 5:1 medium had formed an appressorium than had germinated conidia produced in the other two media. Conidia produced in 15:1 medium were more variable in successfully germinating and forming an appressorium on the leaves of *S. exaltata* than were conidia from 40:1 or 5:1 media (Fisher's equality of variance test; $P = 0.08$ and $P = 0.01$, respectively). Variance of the cellophane membrane germination data for conidia from 15:11 medium was comparable to that calculated for the other conidial types.

**Efficacy of conidia in inciting disease symptoms in *S. exaltata*.** All conidial treatments reduced the plant growth parameters measured when compared with the controls (Table 4). Seedlings inoculated with conidia produced in 5:1 medium had the fewest number of leaves remaining per seedling of any treatment. Conidia produced in 5:1 medium also induced greater losses in shoot dry weights than did conidia from 40:1 medium. Conidia from 15:1 and 5:1 media induced greater reductions in seedling shoot height than did conidia from 40:1 medium.

## DISCUSSION

The culture medium influenced a variety of conidial attributes when conidia of *C. truncatum* were produced in liquid culture. These attributes included conidial size, nuclear number, potential for germination, formation of appressoria on cellophane membranes and leaves of *S. exaltata*, and efficacy in inciting symptoms of disease in *S. exaltata*.

Nutritional conditions and other environmental factors can impact the morphology of spores produced in culture and under natural conditions (18,22). In our study, lowering the C/N ratio

of the conidiation medium while maintaining a constant total carbon concentration resulted in the production of conidia with longer lengths and shorter widths. The influence of the nutritional environment on the size of spores produced has been reported for a variety of ascomycete fungi (14,15,20), but not to our knowledge for a *Colletotrichum* sp.

*Colletotrichum* spp. can differ considerably in the average number of nuclei per conidium, especially when conidia are produced in liquid culture (25). In this study, conidia of *C. truncatum* most frequently contained one nucleus per conidium, although culture medium did differentially influence the proportion of conidia that contained zero or two nuclei per conidium. Approximately 10% of binucleate conidia from 5:1 medium contained a central septum, a condition that could indicate the completion of the sequence of germination events preceding the emergence of a germ tube from conidia of *C. truncatum* (24). This also could partially account for conidia from 5:1 medium germinating more rapidly on cellophane membranes and leaves of *S. exaltata* than the other conidial types. Binucleate conidia (5:1) that did not possess a septum may not yet have formed one, or may have resulted from nucleus distribution errors during conidiogenesis.

A greater proportion of conidia produced in 5:1 medium germinated and produced an appressorium on cellophane membranes or on leaves of *S. exaltata* than conidia produced in the other two media. The germination of conidia of *Monilinia fructicola* also was influenced by the medium used to produce conidia (21). Differences in the quantity and types of endogenous compounds within the different conidial types may partially account for the germination responses seen. In fact, when examined microscopically, conidia from 40:1 medium were more refractile than conidia from the other treatments, presumably because of an abundance of lipid-filled vesicles. The quickness of conidia from 5:1 medium to form appressoria may be due to these conidia containing only minimal energy storage reserves. The lipid content of conidia produced in 5:1 medium is lower than for conidia from 15:1 and 40:1 media on a dry weight basis (M. A. Jackson, *unpublished results*). Researchers have demonstrated that only after excess nutrients were used did germinated conidia of *C. acutatum* successfully form appressoria (2).

Endogenous inhibitors and extracellular matrix have been implicated in influencing conidial germination for several species of *Colletotrichum* (10,12,13,17). The importance of these substances in differentially influencing the germination of conidia produced in our study is unknown.

A higher percentage of conidia produced in 15:1 medium germinated on leaves of *S. exaltata* than on cellophane membranes. Leaf surfaces can provide nutrients that overcome spore germination inhibitors (11). Microbiological or chemical environments on the leaf surface also could have contributed to the breakdown or inactivation of spore germination inhibitors (1,19). Phylloplane microorganisms and exudates may have contributed to a greater portion of conidia from 5:1 medium forming appressoria on leaves rather than on cellophane membranes (2,8).

Conidia produced in 15:1 medium were more variable in

TABLE 2. Influence of the nutritional environment during conidiation of *Colletotrichum truncatum* on the frequency of conidia that germinated and formed appressoria on cellophane membranes

| Carbon/nitrogen ratio of conidiation medium | Germination and appressoria formation (%) after time on membrane | | | | | |
|---|---|---|---|---|---|---|
| | 3 hr | | 6 hr | | 12 hr | |
| | $G^x$ | $G + A^y$ | G | G + A | G | G + A |
| 40:1 | 25 a$^z$ | 0 a | 35 b | 2 b | 39 b | 8 b |
| 15:1 | 5 b | 0 a | 18 c | 1 b | 13 c | 2 c |
| 5:1 | 48 c | 1 a | 69 a | 14 a | 76 a | 30 a |
| LSD | 7 | ... | 10 | 5 | 9 | 4 |

[x] Germinated conidia.
[y] Conidia that germinated and formed appressoria.
[z] Values within columns followed by the same letter are not significantly different ($P \leq 0.01$).

TABLE 3. Influence of the nutritional environment during conidiation of *Colletotrichum truncatum* on the frequency of conidia that germinated and formed appressoria on leaves of *Sesbania exaltata*

| Carbon/nitrogen ratio of conidiation medium | Germination and appressoria formation (%) after time on leaf surface | | | |
|---|---|---|---|---|
| | 6 hr | | 24 hr | |
| | $G^x$ | $G + A^y$ | G | G + A |
| 40:1 | 33 b$^z$ | 10 c | 50 b | 40 b |
| 15:1 | 37 b | 24 b | 59 b | 54 b |
| 5:1 | 76 a | 61 a | 82 a | 70 a |
| LSD | 11 | 11 | 15 | 14 |

[x] Germinated conidia.
[y] Conidia that germinated and formed appressoria.
[z] Values within columns followed by the same letter are not significantly different ($P \leq 0.01$).

TABLE 4. Comparison of growth measurements of seedlings of *Sesbania exaltata* inoculated with conidia of *Colletotrichum truncatum* produced in liquid media of differing nutritional environments$^y$

| Carbon/nitrogen ratio of conidiation medium | Top height (cm) | Shoot dry weight (mg) | Leaves per plant |
|---|---|---|---|
| 40:1 | 9.4 b$^z$ | 29 b | 2.3 b |
| 15:1 | 7.2 c | 22 bc | 1.7 b |
| 5:1 | 6.4 c | 14 c | 0.7 c |
| Control | 11.8 a | 77 a | 5.8 a |
| LSD | 2.2 | 13 | 1.0 |

[y] Measurements made on 29-day-old seedlings of *S. exaltata* 8 days after inoculation with conidia of *C. truncatum*.
[z] Values within columns followed by the same letter are not significantly different ($P \leq 0.01$).

germination and appressorial formation on leaves of *S. exaltata* than were conidia from 40:1 and 5:1 media, but variances were not significantly different when germination was assayed on cellophane membranes. Conidia from 15:1 medium apparently were influenced more than the other conidial types by the nutritionally and microbiologically variable environment present on leaf surfaces. Understanding how to consistently foster microbial and nutritional environments in the phyllosphere that favor germination and appressorial formation would contribute significantly to our understanding of how to reliably control weeds with mycoherbicides in the field.

Conidia produced in 5:1 medium were the most effective, whereas conidia from 15:1 medium were intermediate in reducing plant growth parameters. Success in forming appressoria frequently was associated with conidial efficacy in inciting symptoms of disease. Limiting dew to 6 hr would likely have increased the relative efficacy of conidia from 5:1 medium over conidia from 15:1 medium, because conidia produced in 5:1 medium were comparatively even more successful than conidia from 15:1 medium in forming appressoria after 6 hr. The requirement for an extended period of dew formation to achieve infection of weeds is a constraint to the commercial development of many mycoherbicides (26). We have demonstrated that the impact of this constraint could be reduced by developing media in which conidia with improved rates of germination and appressorial formation are produced. The development of spore production protocols that optimize conidial numbers, germination and infection rates, and resistance to viability loss during formulation (7) would enhance the likelihood of successfully developing *C. truncatum* as a bioherbicide agent against *S. exaltata*.

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