Environmental Factors Influencing the Formation of Basidia and Basidiospores in *Thanatephorus cucumeris*

Gerard C. Adams, Jr., and Edward E. Butler

Research assistant and professor, respectively, Department of Plant Pathology, University of California, Davis 95616.
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ABSTRACT

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Isolates of *Thanatephorus cucumeris,* anastomosis group (AG) AG-1 and AG-4, each capable of sporulation on agar media, were grown in different environments. The influence of aeration, relative humidity (RH), carbon dioxide (CO_2) concentration, and substrate drying on formation of basidia and basidiospores was analyzed. Fruiting occurred in 3-L chambers, containing 15 cultures each in a 15 × 100-mm petri plate, only when ventilation was adequate. Isolates of AG-1 formed basidia when air (99% RH) circulated through chambers at 20 L/hr while AG-4 isolates required 45 L/hr. Incubating cultures in the presence of alkali and in CO_2-enriched environments suggested that the accumulation of respiratory CO_2 within chambers receiving air flow rates <45 L/hr inhibited fruiting. Isolates of AG-1 formed basidia at 0.06%, but not at 0.10%, CO_2 while AG-4 isolates did not form basidia at CO_2 concentrations above 0.03%. Incubation of cultures in different relative humidities had little effect on fruiting unless low RH caused agar medium to visibly shrink from evaporative loss of water. Isolates of AG-1 were stimulated to produce abundant basidia by slow drying of the sporulation medium while AG-4 isolates were unaffected. Although isolates of AG-1 usually did not produce basidia after growth on media containing <1.0 g of $NaNO_3$ per liter, drying of the sporulation medium superseded the influence of nitrogen nutrition.

Thanatephorus cucumeris (Frank) Donk (anamorph: *Rhizoctonia solani* Kühn) is known to produce basidia and basidiospores in culture. Fruiting has been induced by several methods (11,14), but results have been unreliable and unpredictable. Yet, research seldom has been directed toward defining or controlling the many environmental variables that might influence fruiting. However, as early as 1929, Kotila (7) suggested that aeration of cultures of *T. cucumeris* improved fruiting, and he stated that relative humidity (RH) was the controlling factor in fruiting when other conditions were favorable. Later, Whitney (13) also suggested that aeration was important. It is known that inadequate aeration alters the development of other fungi (5,12). The sporulation of *Athelia rolfsii* (Curzi) Tu and Kimbrough (anamorph: *Sclerotium rolfsii* Sacc.), a fungus with fruiting habits similar to *T. cucumeris,* was favored by constant aeration and drier air was superior to moist (4). Inadequate ventilation of cultures caused abnormal pilei in *Polyporus brumalis* (Pers.) Fries (9), inhibited cap expansion in *Collybia velutipes* (Curt, ex Fries) Quel (9), and severely restricted the fruiting process in *Schizophyllum commune* Fr. (8). Inhibition of the fruiting process by lack of ventilation could be due to the accumulation of respiratory carbon dioxide (CO_2), production of ethylene or other volatile gases, or excessive RH. No previous investigations have successfully analyzed the influence of these factors on fruiting of *T. cucumeris.*

The present paper examines the effects of aeration, RH, and drying of the substrate on sporulation. It is part of a larger study examining the influence of environment, nutrition, and genetics on fruiting in *T. cucumeris.* Our goal was to define conditions for precise control of the formation of the sexual state of *R. solani.*

MATERIALS AND METHODS

Four isolates of *T. cucumeris* known to fruit on agar medium were used: 43 and 239, anastomosis group one (AG-1) (1), and 608 and 610 AG-4 (2). They were maintained on potato-dextrose agar (PDA) and transferred to glucose-nitrate agar (GNA) (2) for each experiment. The GNA (20 ml) was in one compartment of a two-compartment petri plate. The other compartment contained 10 ml of salts-thiamine agar (STA), a medium lacking glucose or nitrate (2). After 2–3 days on GNA the isolates grew into the compartment containing STA. Basidia formed on STA on the seventh to 10th day of incubation when conditions were favorable for fruiting.

The amount of sodium nitrate in the GNA was varied with respect to isolate and experiment. Isolates of AG-4 were grown on GNA with 0.25 g of $NaNO_3$/20 g of glucose per liter and AG-1 isolates with 6 g of $NaNO_3$/20 g glucose per liter. However, during experiments in which the effect of drying of the medium on sporulation was measured, AG-1 isolates were grown on GNA with 1.0 g of $NaNO_3$ per L. Measurement of the amount of sporulation occurring in each treatment was used to compare treatments in all experiments. Sporulation was quantified by counting the total number of spores discharged from a plate during 24 hr, as previously described (2).

Rate of airflow. Isolates were transferred to GNA and following 2–3 days growth, plate lids were removed and the plates were placed randomly in 3-L plastic chambers (26 × 19 × 6 cm) and incubated for 10 days. Each chamber contained 15 plates on shelves constructed of hardware cloth (6 × 6-mm mesh) (Fig. 1). During experiments on aeration, flowing moist air was uniformly distributed within the chambers by a rigid plastic tube (7 mm i.d.) that was perforated with holes (0.5 mm in diameter and 1 cm apart) and molded to fit against the inside walls (Fig. 1). Each wall contained a 1.0-cm-diameter exit hole covered with a Millipore filter (0.45-μm pore size). Air, from a source fitted with a compressed air regulator, was passed through an airstone submerged in 12 L of glass distilled water (gdw) and into a flowboard where flow rates were adjusted by utilizing calibrated glass capillaries of various sizes, and a constant pressure head was maintained by a 60-cm water column. The formation of basidia and basidispores was examined at airflows of 0, 20, 45, 60, and 90 L/hr.

Influence of carbon dioxide. The rate of airflow controlled the rate of removal of respired gases and the replacement of oxygen within the chambers. However, to examine the direct influence of CO_2 concentration on basidial formation, cultures were exposed for 10 days to air with 0.03, 0.06, 0.10, and 0.20% CO_2 flowing at 60 L/hr. Compressed CO_2, in a balance of air, and air were separately passed through gdw then blended by flowboards to obtain desired CO_2 concentrations. The resulting gas mixtures were sampled by syringe prior to entering the chambers. The percentages of CO_2, O_2, and N_2 were analyzed by gas chromatography. Additional cultures were placed in two sealed chambers without airflow; one chamber

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was sampled to determine the levels of respiratory CO_2 that accumulated during the 10-day period and the other contained filter paper saturated with 100 ml of 10 M KOH to remove CO_2.

Relative humidity. Airflows of desired relative humidities (RHs) were obtained by passage of air through water bubblers and then through saturated salt solutions with excess of undissolved solute (6). This reduced the RH of the air to the equilibrium RHs over the solutions and permitted absorption of the air's water vapor without alteration of the equilibrium RH over the solutions. The saturated salt solutions had the following equilibrium RH values at 20 C: $NaNO_2$, 66%; $(NH_4)_2SO_4$, 80.6%; $NH_4H_2PO_4$, 93%; and $CuSO_4$, 98% RH (3); and gdw provided 100% RH. Air was passed through gdw, into two 1-L bottles each containing 700 ml of a saturated salt, through a flow meter, and into a chamber containing the cultures. The cultures and the solutions were incubated at 20 ± x C. In addition, the solutions were placed in a tub of water to buffer temperature fluctuations. Such fluctuations were monitored by a recording thermograph. Air flow rate (60 L/hr) was monitored and controlled independently at each chamber by a flow meter and a needle valve.

The RH actually obtained within each chamber was determined by measuring the water loss or gain by three $CaCl_2$ solutions of known equilibrium RH (10) above, equal to, and below the RH of the air flowing into each chamber. Each of the three solutions were added to four 15 × 50 mm dishes, 10 ml/dish, and the 12 dishes were incubated, along with cultures, within a chamber for 10 days. The solutions were weighed prior to and after incubation. The gain or loss of water by each $CaCl_2$ solution in a chamber was plotted as a function of its original RH. A line of best fit was determined for the data by linear regression. The RH within each chamber was determined by extrapolation to the abscissa (Fig. 2). The RH at this point on the abscissa is considered to be the average RH within the chamber since it represents the equilibrium RH of a $CaCl_2$ solution that would neither gain nor lose water during the experiment.

Effects of drying of the substrate on sporulation. Isolates of AG-1 and AG-4 were placed in chambers that received airflows of 60, 90, 120, and 180 L/hr at ~99% RH. The amount of water lost from plates after 10 days of incubation was determined gravimetrically for the whole plate and for the medium on the sporulation side only. The amount of sporulation in each plate in response to water loss by that plate was graphed.

RESULTS

Influence of airflow rate. Isolates of AG-1 and AG-4 did not fruit if no air flowed through the chambers in which cultures were enclosed. Isolates of AG-1 fruited sparsely and formed many abnormal basidia at an airflow rate of 20 L/hr. The concentration of CO_2 in chambers receiving this flow rate reached 0.06% by the third day of incubation. Isolates of AG-4 and AG-1 formed abundant basidia at airflow rates of 45 L/hr and fruiting at 60 and 90 L/hr was not significantly different from that at 45 L/hr for either group.

Influence of carbon dioxide. When the CO_2 concentration of the incoming air was varied, isolates of AG-4 did not fruit at CO_2 levels higher than atmospheric (0.03%). Isolates of AG-1 appeared to be more tolerant and fruited at 0.06%, but not at 0.10% CO_2. After 10 days of incubation in CO_2-enriched atmospheres, isolates of AG-4 transferred to 0.03% CO_2 did not recover the ability to sporulate. Isolates of AG-1, however, formed minute clusters of new basidial cymes and fruited sparsely within 10 days of transfer. In the absence of airflow, chambers containing cultures accumulated CO_2, reaching 6.2% after 6 days of incubation. The cultures did not fruit and aerial hyphae, which were not present in cultures in flowing air, covered the sporulation media. Aerial hyphae may have arisen in response to 100% RH because the RH of flowing air was measured at 99% RH. In sealed chambers containing filter paper saturated with 10 M KOH, cultures of both groups fruited and aerial hyphae were abundant. The same amount of 10 M KOH placed in a beaker within a chamber was less effective in removing CO_2.

Influence of RH. After 10 days at flow rates of 60 L/hr, the average RH values in the chambers containing cultures were 99, 97.7, 93.5, 86.7, and 85.3%; the expected values would have been 100 (H_2O), 98 ($CuSO_4$), 93 ($NH_4H_2PO_4$), 80.6 ($(NH_4)_2SO_4$), and 66% ($NaNO_2$), respectively (Fig. 2). During this period, diurnal temperature fluctuations in the incubator averaged ± 0.54 C with a maximum of ± 0.70 C. The numbers of basidia formed by cultures of AG-1 and AG-4 incubated in 99, 97.7, or 93.5% RH were uniformly high and not significantly different. At 86.7 and 85.3% RH, the sporulation medium dried excessively, which prevented formation of basidia. Discharged basidiospores from isolates of AG-1 were sparse (but evident) on agar surfaces surrounding basidial cymes at 99 and 97.7% RH, but not at lower RHs. Basidiospores from isolates of AG-4 were not evident in any chamber receiving airflow, although basidia were abundant. Within hours after removal from chambers, sterigmata formed and spores were discharged when cultures of AG-4 and AG-1 were inverted over plates of water or agar. This suggests that either 100% RH and/or relatively still air conditions were optimal for formation of sterigmata and basidiospores, although basidia form abundantly at the airflow rates and RHs previously mentioned.

Influence of drying of the substrate on sporulation. Isolates of AG-1 after growth on GNA with 1.0 g of $NaNO_3$ per liter

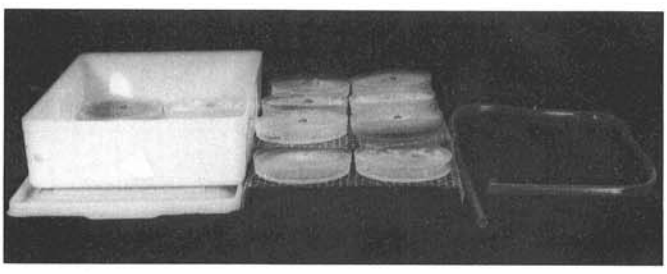

Fig. 1. Fifteen cultures in petri plates without lids are arranged on wire shelves, enclosed, and incubated for 10 days in the plastic chamber below. The perforated plastic tube serves to distribute air throughout the closed chamber. Air escapes through four holes cut into the walls of the chamber and covered with Millipore filters.

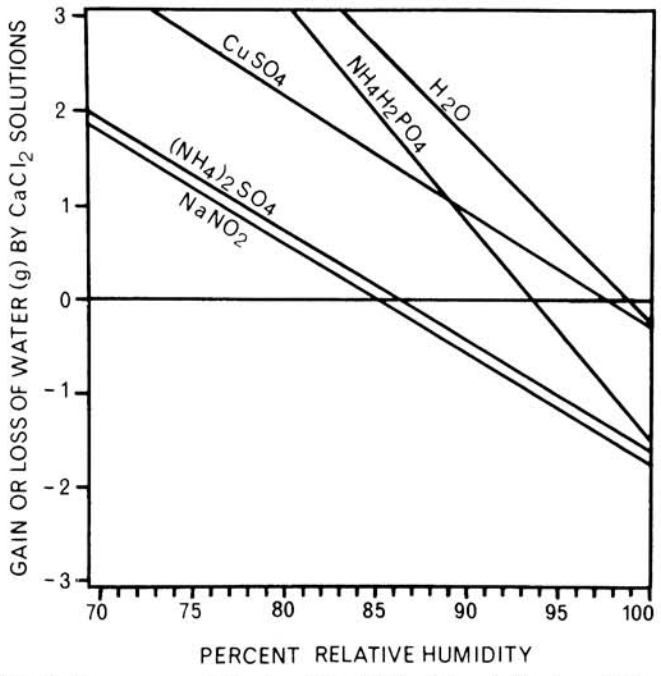

Fig. 2. The average relative humidity (RH) of the air flowing within a certain chamber is indicated by the point where one of the lines crosses the abscissa. On that line is recorded the equilibrium RH of the saturated salt solution through which moist air was passed to obtain the measured RH. The RH was determined by measuring the loss or gain of water by three $CaCl_2$ solutions of known equilibrium RHs weighed prior to and after 10 days of incubation within a chamber.

sporulated profusely on sporulation media that had become visibly shrunken, though not excessively dried, due to water loss. Total spore discharge by these cultures increased linearly ($y = -5{,}449.3 + 5{,}667.6\,x$, $r = 0.82$) with increasing water loss (1–8 g) by the 10 g of sporulation medium in each plate (Fig. 3). Isolates of AG-1 normally fruited poorly after growth on GNA containing 1.0 g of $NaNO_3$ per liter (2). However, drying of the sporulation media (STA) induced sporulation at levels of nitrogen equivalent to that occurring after growth on GNA with high nitrogen levels (3–6 g of $NaNO_3$ per liter) without drying. Drying of the medium on the growth side of partitioned petri plates supporting rapid growth of mycelium had no influence on sporulation. Media on the sporulation side of the partitioned petri dishes, which dried excessively (beyond 8 g of water loss), did not provide an adequate substrate for formation of basidia.

DISCUSSION

Obtaining and controlling the formation of basidia and basidiospores of *T. cucumeris* required the characterization of the following interdependent environmental variables: RH and rate of flow of air over cultures and their influence on the buildup of respiratory CO_2 and evaporative loss of water from the sporulation medium. Control of each of these environmental factors coupled with a method of quantifying sporulation enabled discovery of the unique responses of isolates of AG-1 and AG-4.

Isolates of AG-1 formed abundant basidia in response to evaporative loss of water from the sporulation medium even when the fungi had grown with $NaNO_3$ concentrations too low to permit fruiting on fully hydrated sporulation medium (2). In contrast, dehydration had no measurable influence on AG-4 isolates.

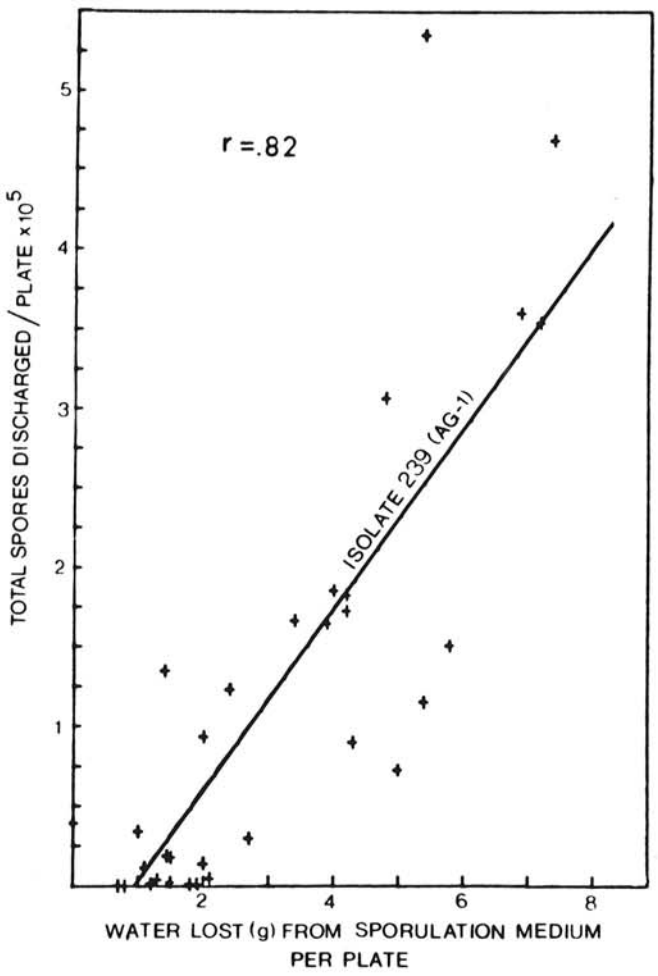

Fig. 3. Numbers of spores discharged by *Thanatephorus cucumeris* isolate 239 (AG-1) increased in response to drying of the sporulation medium. The numbers of spores discharged by each plate, during 24 hr after removal from incubation chambers, was plotted as a function of the water lost, during 10 days of incubation, by the 10 g of sporulation medium originally in each plate.

Evaporative loss of water from agar would be affected by flow rate and RH of air passing through the chamber. However, the measurement of these affects on evaporative loss of water was masked by nonuniform drying within a chamber. Water loss was partly related to the proximity of cultures to exit holes in the walls of the chambers. Air currents exiting through the holes could rapidly dry one culture while having little effect on another nearby. A more uniform drying perhaps could be achieved if there were more exit holes in the walls of the chambers. However, this might not affect such a change because air eddies interacting with the walls of petri plates also determined what area of a culture would shrink from drying.

The air eddy effects are apparently responsible for a lack of uniform sporulation over the entire surface of the medium by AG-1 isolates. Air eddies increased dehydration and caused areas of reduced agar thickness ranging from 27 to 55% of the thickness of surrounding agar in the same compartment of a plate. Sporulation was most abundant in these shrunken areas. There was a suggestion of a similar influence on AG-4 isolates, but it could not be substantiated by measurement. Generally isolates of AG-4 sporulated uniformly over the surface of the sporulation medium.

RHs $<$ 100% not only stimulated sporulation in AG-1 by drying the substrate, but also favored formation of basidia in isolates of both groups by preventing growth of aerial hyphae and the buildup of condensation. At 100% RH, aerial hyphae covered basidial cymes and condensation formed on the hyphae. Condensation occurring in close proximity to basidia caused basidia to abort and form hyaline monilioid cells.

Flowing air, in addition to flushing chambers of accumulating respiratory CO_2, temporarily prevented or suppressed sterigmata and basidiospore formation. Still air was required for optimum sporulation. Further study will be needed to understand the significance of this phenomenon. In this study, sporulation ceased after 3 days in still air at 100% RH and aerial hyphae formed. Probably, a more extended period of sporulation would occur in nature where air movement and relative humidity fluctuate diurnally. Alternatively, hymenia may form in a more indeterminant manner in the field than on agar media.

The results showed that AG-1 and AG-4 isolates were not tolerant of CO_2 concentrations much above atmospheric levels. This was in accord with our past experience in attempting to induce fruiting in these isolates. Sporulation generally was very sparse and occurred only near the inner wall in covered petri plates. Such cultures fruited better on lab benches, where air passage was greater, than in incubators or when stacked in open containers. It is difficult to speculate on how differences in CO_2 tolerance between AG-1 and AG-4 isolates would be reflected in differences in the life histories of the two groups. However, because isolates of AG-1 were grown on a medium containing much higher concentrations of $NaNO_3$ than were the AG-4 isolates, differences in tolerance of CO_2 might have been influenced by differences in growth rate and metabolism. Regardless, intolerance to CO_2 much above atmospheric levels assures that sporulation will not occur in soil where dispersal of spores would be precluded.

It is not known whether most isolates of AG-1 and AG-4 will exhibit the same responses to the environment as reported here. However, the results suggest that anastomosis grouping has separated biologically different isolates.

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