MODULATION OF SPORULATION OF \textit{ALTERNARIA TAGETICA} BY CARBON DIOXIDE\(^1\)

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\textbf{ABSTRACT}

A mutant of \textit{Alternaria tagetica} with altered regulation of sporulation was obtained. Wildtype strains require light to sporulate whereas the mutant sporulated in the absence of light. Sporulation of the mutant was unaffected by culture plate sealing which inhibited the wildtype 99-100%. Absorption of CO\(_2\) from the atmosphere within the plates via KOH counteracted wildtype inhibition by plate sealing and induced light-independent sporulation in wildtype strains of \textit{A. tagetica}, as well as in \textit{A. carthami}, \textit{A. macrospora}, \textit{A. porri}, and \textit{A. cucumerina}, species which typically require light for sporulation. Continuous fluorescent light inhibited sporulation for all wildtype \textit{Alternaria} species tested at both 28 C and 20 C, but sporulation was induced under the same light regime when fungi were grown 3 days at 28 C followed by 3 days at 20 C. The identical temperature drop induced sporulation in the dark in \textit{A. tagetica}, \textit{A. cucumerina}, \textit{A. macrospora}, and \textit{A. carthami}. Both mutant and wildtype strains of \textit{A. tagetica} produced 7-8 times more CO\(_2\) on potato dextrose agar (PDA) than on V-8 agar. However, the mutant produced more conidia on PDA than on V-8 whereas the wildtype produced fewer conidia on PDA. Many aspects of \textit{in vitro} sporulation may be attributable to the influence of CO\(_2\).

Key Words: nutrition, light, respiration, carbon dioxide.

Sporulation of many \textit{Alternaria} species has precise requirements (12, 20, 23). Many species require exposure to light followed by darkness to produce conidia in culture (17, 29, 31). However, some plant pathogenic species that require light in culture produce conidia in constant dark \textit{in vivo}, although discontinuous light stimulates the process (24). Desiccation and nutrient depletion stimulate sporulation \textit{in vivo} and \textit{in vitro} (24).

Light stimulates conidiophore formation but inhibits conidial development in light-dependent species; therefore, sporulation may be inhibited by either continuous dark or continuous light (17, 29, 31). The action spectra of light inhibition and stimulation of sporulation have been described in detail for a number of species (12, 16). However, the mechanism by which light modulates this process has remained elusive. Sporulation of \textit{Alternaria tagetica} Shome and Mustafee, a pathogen of marigold (\textit{Tagetes} species), is light dependent (5). While working with \textit{A. tagetica} I discovered a mutant which produces conidia in the absence of light. The objective of this investigation was to determine characteristics of the mutant which may have resulted in light-independent sporulation. In the process it was discovered that carbon dioxide plays a role in the regulation of sporulation in the genus \textit{Alternaria}. A preliminary report has been published (3).

\textbf{MATERIALS AND METHODS}

\textbf{Cultures.—}Origins of \textit{A. tagetica} isolates 17, ATCC 58179 and 58771, \textit{A. carthami} Chowdhury ATCC 58170, \textit{A. citri} Ell. and Pierce ATCC 58171, \textit{A. macrospora} Zimm. ATCC 58173, \textit{A. porri} (Ellis) Cif. ATCC 58175, and \textit{A. raphani} Groves and Skolko 58176 were previously described (4). \textit{A. cucumerina} (Ell. and Ev.) J. A. Elliott, isolate CU-4, from \textit{Cucumis sativis} L. was collected in Tucson, Arizona in 1983 and \textit{A. brassicaceae} (Berk.) Sacc., isolate RB-1, from \textit{Brassica oleracea} L. was collected in Maricopa County, Arizona in 1984. Stock cultures were maintained at 26 C under 11,500 ± 700 lux coolwhite fluorescent light in 9-cm plastic Petri dishes containing 20 ml of 3% V-8 vegetable juice, 2% agar (5/2 agar). For long-term storage 3-mm plugs of sporulating cultures were maintained at room temperature in 25-ml vials containing 5 ml of sterile distilled water. Illuminance was measured.
with a model 765 Weston sunlight illumination meter (Weston Instruments Inc., Newark, New Jersey).

Dark-sporulating strains of *A. tagetica* (ATCC 58179) (Fig. 1) were obtained from 2 spores borne on a mycelial tuft which arose spontaneously in a dark-grown culture on 5/2 agar at 27°C. A dark-sporulating mutant of *A. carthami* Chowdhury (ATCC 58170) (Fig. 1) arose as a round sector (2 mm diam) bearing about 20 spores on 5/2 agar kept under continuous 11,500 lux coolwhite fluorescent light at 20°C. Both mutants and wildtypes were transferred by single spores twice serially before being used in experiments. Mutant and wildtype conidia produced on 5/2 agar were morphologically indistinguishable.

**Quantification of sporulation.**—To quantify spore production the diameter of a given culture was first measured. The culture plate was then flooded with about 10 ml of 0.01% Triton X-100 and spores were dislodged by thoroughly rubbing the agar surface. Plates were washed three times and the washings were pooled and diluted to an appropriate spore concentration (200–2000 spores/ml). The spore suspensions were stirred with stir bars while ten, 10-μl samples were withdrawn with a micropipette. The total number of spores in each 10-μl sample was enumerated under a dissecting scope (45×). Treatments were replicated 2 to 4 times and experiments performed at least twice.

**Plate sealing.**—To determine if sporulation of various fungi is influenced by sealing culture plates, plastic culture plates containing 20 ml 5/2 agar were sealed tightly shut after seeding by wrapping the edges with 3 layers of Parafilm. Cultures were incubated for 5 days at 26°C under a 12-h diurnal light. In a few experiments, the plates were sealed with plastic tape to rule out the possibility that observed phenomena were specific for Parafilm.

**Effect of CO₂ on sporulation.**—To test the effect of volatiles within sealed plates on fungus sporulation, two sterile polypropylene centrifuge tube caps (24 mm inner diam, 9 mm inner height) were set (open-end up) in the medium of each plate. To remove volatiles either 2 ml of 20% potassium hydroxide (27) or 1 g of activated charcoal (19) was added to each cap. Plates were seeded with 3-mm diam agar plugs of 5–8-day-old cultures between caps and sealed with Parafilm as above. Parafilm has a CO₂ permeability of 400 cc/m²/24 hr at 23°C and 50% RH (American Can Company, Greenwich, Connecticut 06830). Sporulation was quantified after incubation at 27°C for 5–8 days either under 12 h diurnal light (11,500 lux) or in the dark.

In other experiments, various CO₂ levels were maintained in similar sealed culture plates containing 5/2 agar by adding 2 ml of either Pardee's CO₂ buffer (PB) (22) or 20% KOH to the cups described above. Prior to sealing, the plates were seeded with 3-mm diam plugs of 5–8-day-old cultures. After incubation at 27°C for 5 days under a 12-h diurnal light cycle the quantity of conidia produced in each CO₂ level was determined. The concentration of CO₂ maintained above the buffers was determined by incubating 25-ml vials with open-top caps and teflon faced silica septa (Fischer Scientific, Springfield, New Jersey 07081) containing 4 ml of a buffer simultaneously with the treatments. Three milliliter air samples were withdrawn from the vials by plastic syringe periodically and the concentration of CO₂ was determined with a Beckman model 865 infrared gas analyzer according to Clegg et al. (2). The CO₂ levels tested were 0.009% (KOH), 0.039% (PB), 0.050% (Ambient), 0.117% (PB), and 0.233% (PB).

**Effect of medium.**—Cotty and Misaghi (5) reported that *A. tagetica* sporulated less on potato dextrose agar (PDA) than on 5/2 agar. To determine if this disparity may be due to differences in CO₂ evolution, spor and CO₂ production were quantified on the two media. Carbon dioxide
evolution was quantified in the closed system described by Foutz et al. (9). The mutant and the wildtype were grown on PDA and 5/2 agar for 5 days at 26 °C under the diurnal light cycle. On the fifth day, after a 5-8 h period of light, cultures were placed in a glass chamber connected by tubing in a closed loop to an infrared gas analyzer (Beckman model 865). Air was passed through the loop with a diaphragm pump at 5.2 L/min allowing the air to circulate between the chamber and the analyzer as previously described (9). Carbon dioxide evolution of each culture was monitored continuously at 28 °C for 5 min in the dark. Treatments were replicated 3 times and the experiment was performed twice. Conidia were enumerated after each experiment as previously described.

Effect of temperature drops. — Alternaria taegetica isolates were grown for 5 days on 5/2 agar both under continuous illumination (11,500 lux) and in darkness at both 20 and 28 °C. These isolates were also grown at 28 °C for 3 days and then at 20 °C for 2 days both under continuous illumination and in darkness. Cultures were evaluated microscopically (45 x) for spore production at the end of each experiment.

Pathogenicity. — The pathogenicities of A. taegetica wildtype and mutant spores produced under 12-h diurnal light and mutant spores produced in constant dark were determined as previously described (6). Marigold plants (21-28 days old) were sprayed to runoff with a 0.01% Triton X-100 solution containing 800-1000 spores per ml and placed in a dark humidity chamber at 28 °C for 24 h. Plants were then maintained at 28-30 °C under 12 h of 5800 lux coolwhite fluorescent light daily for 7 days and then ranked and rated.

RESULTS AND DISCUSSION

The mutant of A. taegetica sporulated equally well on 5/2 agar in either continuous dark or a 12-h diurnal light cycle, whereas the wildtype from which it was derived sporulated only in the diurnal light cycle (Fig. 1). Many Alternaria species require light to produce conidiophores and dark to produce conidia (17, 29, 31). The mutant, but not the wildtype, produced conidiophores in the absence of light. Sealing culture plates with either Parafilm or plastic tape resulted in reductions in wildtype spore yield of 99-100% after 5 days on 5/2 agar under 12 h of light daily, but had no effect on sporulation of the mutant. Sporulation of the wildtype, but not that of the mutant, was prevented when mutant and wildtype were grown under 12 h diurnal light in the same sealed plate. Both the wildtype and mutant sporulated abundantly if such plates were left unsealed. The mutant was thus insensitive to light.
COTTY: *ALTERNARIA* SPORULATION

511

...to a volatile product which inhibited the wild-type.

Wildtype inhibition was eliminated by removal of CO₂ from the sealed plates via absorption with 20% KOH (TABLE I). Activated charcoal had no effect. All three wildtype *A. tagetica* strains were induced to sporulate in constant dark at 27 C on 5/2 agar by removing CO₂ from culture plates. Wildtype strain ATCC 58179 produced 948 ± 336 spores/cm² in four such tests. Removal of CO₂ had no effect on mutant spore yield (TABLE I). Sporulation of the wildtype, but not of the mutant, decreased as CO₂ increased from 0.009 to 0.117% (P = 0.001, r = 0.88) and was totally inhibited at 0.233% (FIG. 2). It seems, therefore, that the ability of the mutant to sporulate in constant dark may be due to its reduced sensitivity to CO₂.

Although light-induced rhythmic growth of the wildtype, manifested as growth rings, also was repressed by plate sealing and expressed when CO₂ was removed, radial growth of either strain was not influenced by CO₂.

Sealing culture plates appears to inhibit sporulation of many *Alternaria* species. Wildtype isolates of *A. brassicae*, *A. carthami*, *A. cucumerina*, *A. macrospora*, *A. porri* and *A. raphani* were inhibited 98-100% by plate sealing. *A. citri* was inhibited 83 ± 3%. Removal of CO₂ from sealed plates via absorption with KOH stimulated sporulation in all test species. Furthermore, removal of CO₂ induced light-independent sporulation in all species tested which typically require light (*A. carthami*, *A. cucumerina*, *A. macrospora*, *A. porri*, and *A. tagetica*).

Respiratory CO₂ may play an important role in regulating the sporulation of these fungi. This is suggested in induction of dark sporulation in light-requiring species by removal of CO₂, inhibition of light-stimulated sporulation by elevated CO₂ levels, and the occurrence of a CO₂ insensitive mutant with altered regulation of sporulation. Inhibition of fungal spore production by CO₂ is well documented (1, 18, 21).

The general rule that fungus sporulation is induced by starvation or nutrient depletion, first postulated by Klebs in the last century, has been repeatedly demonstrated with diverse genera (7, 10) including the genus *Alternaria* (14, 23). For example, spore production by *A. tagetica* is greatly inhibited on nutrient-rich potato dextrose agar (PDA) as compared to nutrient-low 5/2 agar (5). This inhibition may result from increased CO₂ production. Evolution of CO₂ by both the mutant and wildtype was 7–8 times greater on PDA than on 5/2 agar. However, mutant spore yield significantly increased (P < 0.01 by analysis of variance) on PDA while wildtype yield on PDA was only 4% of that on 5/2 agar (FIG. 3). Thus, the mutant’s insensitivity to CO₂ eliminated the inhibitory effect of the high nutrient medium. Modulation of sporulation by CO₂ may serve, in a similar manner, to direct these heterotrophs toward resource depletion prior to diversion of energy for dissemination through production of asexual spores.

Since respiration rates of many organisms are positively correlated with temperature (11, 25, 31), it was surmised that temperature drops may stimulate conidiogenesis by altering the rate of respiratory CO₂ evolution. *Alternaria tagetica*, *A. macrospora*, *A. cucumerina* and *A. carthami* sporulated neither under continuous illumination nor in constant dark at both 20 and 28 C. However, sporulation was induced in both cases when the temperature was dropped after 3 days from 28 to 20 C. Temperature drops induced sporulation primarily within 5 mm of the growing margins of mycelium existing at the time of the drop. This often resulted in a distinct ring of spores when the plates were evaluated 3 days after the drop.

Numerous intricate techniques to induce or increase sporulation of *Alternaria* species have been developed (20, 23). These include wounding of mycelium (26), desiccation (23), transfer
of mycelial plugs (14), addition of toxicants (8), and other procedures which interfere with normal vegetative development (23). The effectiveness of these techniques may be due to their ability to alter the evolution of respiratory CO₂. Light alters the metabolism and inhibits growth of A. tagetica (5). Techniques to quantify the influence of light on respiration under conditions favoring sporulation are needed in order to examine the relationship between the influences of light and CO₂ on sporulation.

Recent interest in the parameters governing in vitro sporulation of Alternaria species has been spurred by the potential of several species as mycoherbicides (15, 28). Mutants capable of producing conidiophores and conidia independent of light and nutrient depletion and in submerged culture may facilitate the development of this technology. However, to be useful such mutants must be stable and retain their pathogenicity. Mutant and wildtype spores of A. tagetica were equally pathogenic to their host, Tagetes erecta L. The mutant’s characteristics were retained through 10 serial passages on 5/2 agar and no revertants to wildtype were detected among 30,000 spores tested.

During these studies a mutant of Alternaria carthami was obtained which also had light-independent sporulation (Fig. 1) not inhibited by plate sealing. Similar mutants may be inducible for whichever Alternaria species commercial purposes dictate.

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LITERATURE CITED

24. ———, Y. Cohen, and E. Bashi. 1978. Host and
environmental influences on sporulation in vivo. 
physiology. Wadsworth Publishing Co., Bel-
mont, California. 422 p.
ficient technique for inducing profuse sporula-
tion of Alternaria species. Phytopathology 69: 
618–620.
1949. Manometric techniques and tissue me-
227 p.
of Alternaria cassiae for the biocontrol of sick-
lepod (Cassia obtusifolia). Weed Sci. 30: 651– 
654.
29. Witsch, H. V., and F. Wagner. 1955. Beobach-
tungen über den Einfluss des Lichtes auf Mycel-
und Conidienbildung bei Alternaria brassicae 
31. Zimmer, R. C., and W. E. McKeen. 1969. In-
teraction of light and temperature on sporula-
tion of the carrot foliage pathogen Alternaria 

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