Effects of Plastic Film as a Closure Method on Cultural Morphology of Fusarium Species

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ABSTRACT

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Metal-capped test tubes containing cultures of *Fusarium* sp. were sealed with a plastic wrap to control mites. Fungal growth in these tubes was characterized by variation in colony morphology and color and slower growth compared with that of the same isolate in nonsealed test tubes. Variability in spore shape was increased and total spore production was reduced. The atmosphere in sealed test tubes containing cultures of *Fusarium solani* was significantly higher in CO_2 and H_2O and lower in O_2 compared with the atmosphere in tubes not sealed with the plastic wrap.

Additional key words: *Fusarium solani*, carbon dioxide, conidia.

Members of the genus *Fusarium* have an exceptional capacity for physiological and morphological change (2). This capacity has led to the use of standardized conditions when growing and comparing cultures (2, 10). While light, temperature, and nutrient requirements are defined under such standardized conditions, the atmosphere inside the test tube or petri dish is considered ambient, and left undefined. An additional requirement in many laboratories is the control of mite contamination. Mites can be controlled with water or paper traps, with cigarette paper seals (9), by isolation of the cultures, or with a seal of plastic wrap (7). The last three methods have been used routinely in this laboratory.

In an attempt to study the inheritance of selected morphological characteristics of *F. solani* (Mart.) App. et Wr. emend. Snyd. & Hans. the inability to produce a stable cultural growth pattern delayed the investigation. The unstable colony appearance did not occur if the culture tubes were not sealed with "Stretch 'n Seal" (Goodyear Aerospace, Akron, OH 44315, Colgate Palmolive Co., Dist., New York, NY 10022). Several experiments were undertaken to quantify the effect of the sealing procedure and to explain why the changes had taken place.

MATERIALS AND METHODS

All *Fusarium* isolates used were part of the collection of the Fusarium Research Center, including isolates S435 and S219 of *F. solani* received from the Bascom Palmer Eye Institute, Miami, FL 33152.

Fusarium was grown on slants of poato-dextrose agar (PDA) (10) in glass test tubes capped with stainless-steel closures (5). Cultures were sealed with a 3-cm-wide strip of the plastic wrap by stretching the wrap tightly around the holes at the bottom of the cap and the junction between the metal and the glass. Cultures were initiated on the medium with mass transfers of mycelial fragments or single macroconidia before sealing and referred to as "sealed." Test tubes that were capped but not wrapped were referred to as "nonsealed." Cultures were grown 45 cm beneath a series of 40-w fluorescent lights (F40T12/28REL Telepro Lighting, Cherry Hill, NJ 08002) at 25 C (10).

Genetic uniformity of all isolates was increased by growing the parent cultures from a single spore from a colony grown from a single spore (two generations). *Fusarium solani* isolate S435 (S435) was subjected to two additional single spore transfers.

Mass transfers of *F. moniliforme* Sheldon emend. Snyd. & Hans. were made to 24 slants of PDA; 12 were sealed and 12 were left open. Cultures were allowed to grow for 21 days before examination. An identical procedure was followed using *F. lateritium* (Nees.) emend. Snyd. & Hans., *F. oxysporum* Schlect. emend. Snyd. & Hans., *F. rigidiusculum* (Brick) Snyd. & Hans., *F. roseum* (Lk.) emend. Snyd. & Hans., and *F. tricinctum* (Cda.) emend. Snyd. & Hans. Cultures of these species were compared by visual examination.

Fusarium solani was tested with a total of 78 sealed and 78 nonsealed mass transfer cultures and an additional 78 sealed and 78 nonsealed single spored cultures.

Spore counts.—Conidia of S435 were counted using a Coulter Counter Model B (Coulter Electronics, Inc., Hialeah, FL 33011) after 5, 8, 11, 19, and 28 days of growth from a single-spore colony. Ten ml of distilled water were pipetted to the inner wall of the culture tube and the top sealed with Parafilm (American Can Company, Neenah, WI 54956). The tube was slowly

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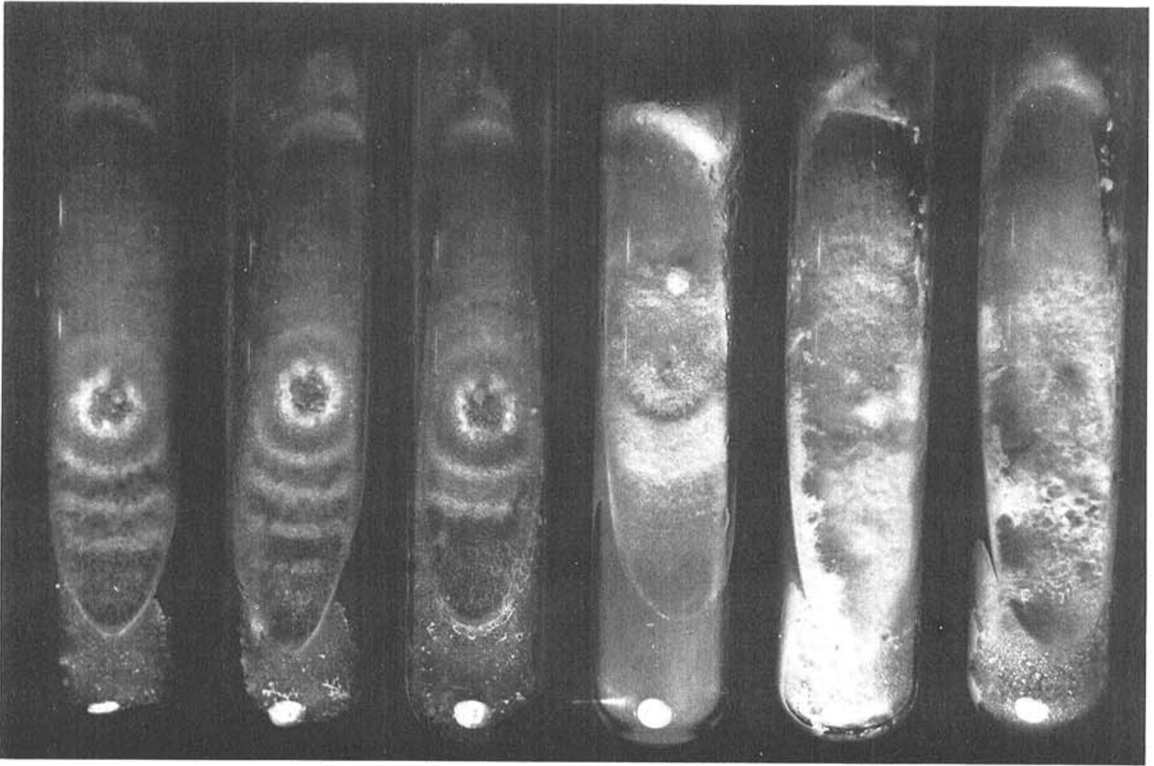

Fig. 1. *Fusarium solani* grown on potato-dextrose agar slants using plastic wrap over metal closure caps to prevent contamination by mites. Three tubes grown with caps but without using wrap seal are shown at the left.

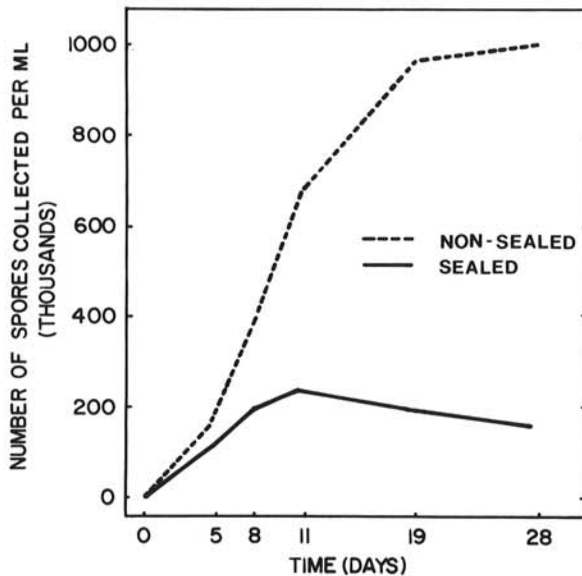

Fig. 2. Spore production by *Fusarium solani* on potato-dextrose agar slants. "Nonsealed" test tubes were covered with a metal cap only, and sealed test tubes were covered with a metal cap and wrapped with plastic wrap. Each point represents the mean spore count (conidia/ml) of spores removed from six test tubes.

inverted twice and the resulting suspension of macro- and microconidia was poured into a separate vessel. Dilutions of the suspension of conidia were made as necessary to reduce counts below 20,000 conidia per ml. Six counts of 1-ml samples were made for each of six sealed and six nonsealed cultures at each time period. An identical procedure was used to compare spore production of single-spore cultures of isolate S219 at 4 and 6 days growth. In both tests, day 1 was the day the spore was transferred to PDA.

Gas analysis.—The amounts of CO_2 and O_2 inside the test tubes containing S435 were determined after 16 days growth from a single spore. A Varian Aerograph Series 1800 gas chromatograph (Varian Associates, Aerograph Div., Walnut Creek, CA 94597) was used following the procedure described by Bollag, Drzymala and Kardos (1). Atmospheres in seven sealed and seven nonsealed test tubes were analyzed from air withdrawn through a rubber septum covering a 3-mm-diameter hole in the top of the metal cap. All test tubes were wrapped with strips of parafilm just prior to sampling to prevent the introduction of outside air.

RESULTS

All isolates, representing seven *Fusarium* species, sensu Snyder and Hansen, were consistently uniform in their respective growth pattern, growth rate, pigment

production, form of sporulation, and appearance when examined after growth on nonsealed PDA slants. However, when grown under sealed conditions, these same isolates were variable in color, growth pattern, and form of sporulation and exhibited slower growth. A typical species, *F. solani* S435, is light-brown in color and sporulates abundantly in a series of concentric rings around the point of transfer (Fig. 1). In contrast, cultures grown in sealed tubes were not uniform but could be classified into groups of similar colony growth characteristics or colony color. Color of the stroma ranged from white to green to shades of tan or a mottle of several of these colors. Mass transfers from any tube showing a single color or color group reproduced the entire range of colors and growth characteristics when grown under sealed conditions. Transfers made from either sealed or nonsealed test tubes and grown in nonsealed tubes were always uniform in color and growth rate.

Spore counts using the Coulter counter were significantly different (Student's *t*-test, $P = 0.01$) between sealed and nonsealed cultures after 5 days (Fig. 2). Significant differences were also found at 4 and 6 days using isolate S219 of *F. solani*. The number of spores produced in open test tubes increased over time while those produced in sealed test tubes reached a plateau after 8 days and remained approximately constant thereafter.

Twelve mass-transfer cultures of S435, were grown on PDA under each of the following conditions: sealed with a cotton plug, sealed with cigarette paper and metal cap, sealed with a metal cap and "Stretch 'n Seal" and covered with a metal cap only. At the same time 12 additional cultures of isolate S435 were grown under each of the above conditions with the addition of a strip (5×3 cm) of "Stretch 'n Seal" placed slightly above the top of the PDA slant inside the tube. This was to allow any volatile coming from the "Stretch 'n Seal" to accumulate inside the test tube but still allow some movement of air. There was no observable difference in growth pattern of *F. solani* cultures grown in tubes with a cotton plug, or sealed with cigarette paper and those sealed with a metal cap only. The insertion of a strip of "Stretch 'n Seal" inside the test tube also had no observable effect on fungal growth.

Carbon dioxide in sealed tubes averaged 3.03 ml/tube (standard deviation = 1.01) while CO_2 in nonsealed tubes averaged 0.184 ml/tube (standard deviation = 0.054). This represented an average of 11.3 and 0.69% CO_2/ml air, respectively. Oxygen levels in nonsealed test tubes were near ambient (18.0-20.8%) while those in sealed test tubes were reduced to 2.03-2.65%.

Mass spectral analysis using an AEI MS 902 High Resolution Double Focus Mass Spectrometer (70 electron volt spectra) on air samples from four nonsealed and four sealed tubes containing S435 after 21 days growth did not reveal any volatiles present in the gas samples. The procedure confirmed the differences in levels of CO_2 and O_2 between sealed and nonsealed condition.

DISCUSSION

The use of plastic films for tube closures was proposed originally because they prevented the drying of growth media (6). However, the permeability of the plastic was never investigated. The growth aberrations reported in this study may have been caused by the accumulation of toxic waste products within the test tube, a decrease in the level of O_2, an increase in the level of CO_2, mutations,or a combination of these factors. Mutation is unlikely, in light of the complete reversibility of the effect when cultures are transferred to nonsealed test tubes. Toxic products may be involved but none was detected by mass spectral analysis. Mass spectral analysis detected isotopes of carbon, nitrogen, and oxygen which occur in relatively low amounts. However, the possibility exists that some other gas not detected by these methods may be involved. Robinson and Park (8) found aldehydes produced by *F. oxysporum* in culture that prevented spore germination or killed spores at low levels. They did not observe any direct effect on hyphal growth.

The elvated levels of CO_2 together with the decreased levels of O_2 in sealed test tubes may be sufficient to account for the reduced growth and spore production. The effect of elevated levels of CO_2 on *Fusarium* has been noted (3, 4). Volatiles from the wrap itself probably are not involved since a strip placed inside the tube did not induce the response. Optimum growth of *Fusarium* cannot be accomplished without attention to the atmosphere in which the cultures are grown. Many methods of culturing fungi involve restricting the atmosphere around the colony and these methods also may result in reduced fungal growth or altered morphology.

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