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Title: EFFECTS OF NEAR-ULTRAVIOLET RADIATION ON CHANGES IN THE BIOCHEMICAL COMPOSITION OF VERTICILLIUM ALBO-ATRUM

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Abstract approved: Dr. W. H. Brandt

This study sought a better understanding of the mechanism of the near-ultraviolet inhibition of microsclerotia and pigment formation in the fungus Verticillium albo-atrum Reinke & Berth. by observing changes which occur in certain major biochemical components: lipids, chitin, RNA, DNA, protein carbohydrates, and pigment.

The most important finding was that dark-reared, microsclerotia-producing cultures generally produced about twice as much total lipid as did UV-reared cultures which produced no microsclerotia.

At least part of the time, the carbohydrate content of the dark-reared cultures exceeded the carbohydrate content of the UV-reared cultures.

The percent apparent protein (measured by the Lowry technique) in the dark-reared cultures declined with age from 25% to 8% by the eleventh day. The apparent protein content of the UV-reared

cultures remained between 17-25%.

No substantial differences in percent chitin and percent DNA of dark-reared and UV-reared cultures were observed.

Effects of Near-Ultraviolet Radiation on Changes in the Biochemical Composition of *Verticillium albo-atrum*

by

George Russell Madarash

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

June 1970

APPROVED:

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Date thesis is presented May 20, 1969

Typed by Mary Jo Stratton for George Russell Madarash

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EFFECTS ON NEAR-ULTRAVIOLET RADIATION ON CHANGES IN THE BIOCHEMICAL COMPOSITION OF *VERTICILLIUM ALBO-ATRUM*

INTRODUCTION

Some isolates of *Verticillium albo-atrum* Reinke and Berth. do not produce microsclerotia or dark pigment when grown in visible or near-ultraviolet radiation (Issac, 1953; Brandt and Wang, 1960; Kaiser, 1962; Leach, 1962; Brandt, 1964a). Factors other than light may augment or reduce microsclerotia and pigment formation. Manganese stimulates pigment and microsclerotia formation (Brandt, 1962). Roth and Brandt (1964) and Kaiser (1964) both found that biotin increases growth and stimulates microsclerotia formation. Some isolates of *Verticillium* require multiple vitamins for maximum microsclerotia formation (Roth and Brandt, 1964). Inositol alone inhibits microsclerotial development, pigment synthesis, and dry weight, whereas inositol together with pyridoxine works synergistically to enhance microsclerotia development and pigment synthesis, and dry weight increase was no longer inhibited (Roth and Brandt, 1964b). Verticillium itself produces a diffusible substance which enhances pigment and microsclerotia formation (Brandt, 1964b). Catechol, when added to the growth medium, caused pigment and microsclerotia to form even in cultures grown under near-ultraviolet radiation (Brandt, 1965).

This study sought a better understanding of the mechanism of the near-ultraviolet effect (mainly inhibition of microsclerotia and pigment formation in Verticillium albo-atrum) by observing the changes which occur in certain major biochemical components: lipids, chitin, RNA, DNA, protein, carbohydrates, and pigment.

MATERIALS AND METHODS

Culturing

Organism

The organism used was a subculture of Verticillium albo-atrum, Reinke and Berth., which was isolated by W. Tolmsoff from a potato grown in soil coming from Klamath Falls, Oregon. After initial growth on 5-21 medium (20 grams sucrose, 3 grams yeast extract, 2.5 grams K_2HPO_4, 2.5 grams KH_2PO_4, and 17 grams agar per liter of medium) (Brandt, 1964a), the cultures were refrigerated. Just prior to this study single spore cultures were obtained. These single-spore cultures served as the inoculum source.

Medium

The medium used in this study was a liquid sucrose-nitrate medium. Each liter contained 20 grams sucrose, 3 grams KNO_3, 0.5 grams $MgSO_4 \cdot 7H_2O$, 1 gram K_2HPO_4, 100 micrograms of thiamine HCl, 2 ml of microelements solution containing the following (Brandt, 1964a): 0.3617 grams $Fe(NO_3)_3 \cdot 9H_2O$, 0.2199 grams $ZnSO_4 \cdot 7H_2O$, 0.1015 grams $MnSO_4 \cdot H_2O$; these were dissolved in 300 ml of distilled water, cleared with not more than two ml of concentrated H_2SO_4 and the solution was brought to 500 ml volume with distilled water (Lilly and Barnett, 1951).

Medium used to culture the fungus in the first experiment was pre-filtered through a nonsterile glass-fiber filter and a nonsterile 1.2μ Millipore filter. The final sterilizing filtration was done during the addition of the medium into the sterile culture flasks by dispensing the medium through a sterile 0.45μ Millipore filter. The Millipore filter holder was mounted on an automatic pipette preset to deliver five ml of medium. During the medium preparation for the first experiment, the 0.45μ Millipore filter became blocked very quickly making it necessary to change filters after each 600 ml. To avoid this problem the medium was subsequently prefiltered through a nonsterile 0.45μ Millipore filter making it possible to filter-sterilize three liters of medium with each sterile filter.

The flasks containing the filter-sterilized medium were incubated at 24°C for at least a week to determine whether contamination had occurred. Water lost by evaporation was replaced with autoclaved distilled water.

Culture Vessels

The fungus cultures were grown in 125 ml Pyrex Erlenmeyer flasks containing 15 ml of medium and closed by preformed polyurethane foam plugs. The plugs were preformed by autoclaving them 15 minutes at 110°C in the end of a 120 x 15 mm test tube.

Preparation of the Inoculum

The inoculum was grown on a rotary shaker for four to six days at 22-25°C in 250 ml Delong flasks with Morton closures, containing 25 ml of sucrose-nitrate medium. The shaker was located in the incubation room. Lighting was available intermittently from overhead fluorescent lamps.

Just prior to inoculation, the contents of each of the shaken cultures was filtered through several layers of sterile cheesecloth layered on the bottom of a sterile Buchner funnel. The filtrate was collected in one sterile flask. The filtrate contained mainly spores. The spore suspension was diluted with sterile distilled water to provide one ml of inoculum per flask. The spore suspension was dispensed to each flask by means of a sterilized automatic pipette preset at one ml.

Physical Conditions

The inoculated flasks were separated into two groups and each group numbered. One was placed in the dark and the second was placed under near-ultraviolet lamps. The dark environment consisted of a metal cabinet entirely free of light except during routine observation. The flasks under near-ultraviolet lights were placed on a metal grating supported two inches above a metal shelf by wooden blocks. Sixty cm above the grating hung four F40 BLB Blacklite

Blue Sylvania lamps (radiation mainly 3200-4000 Å; peak 3650 Å). To obtain uniform irradiation of each of the flasks under the lamps, reflectors were situated above the shelf on four sides. Light intensity readings were made with a Photovolt model 501-M photometer with a U. V. transmitting filter over the photosensor. Readings at 448 different positions on a grid system under the lamps did not have a range of more than 5%. In determining the range, the highest light intensity reading was considered 100%. The temperature range during the first experiment was 22.7-25.5° C in the UV and 23.8-25.6° C in the dark. The temperature range during the second experiment was 22.3-25.0° C in the UV, and 23.9-25.6° C in the dark.

The temperature of each of the incubation areas was monitored continuously with thermographs calibrated with an ASTM mercury thermometer graduated in 0.2° F units.

Harvesting of the Fungus

Collection of the Fungus Material

Harvests during the first experiment were made every 24 hours. During the second experiment harvests were made every 24 hours for the first 72 hours and every 48 hours thereafter.

The cultures to be harvested daily were chosen by means of a random numbers table (Snedecor, 1950). The fungus samples were

collected by filtration of the fungus onto preweighed, numbered, 4.25 cm glass-fiber filters. During the early portion of the growth curve, as many as ten flasks had to be pooled to provide quantitative measurements. Three separate pooled samples of fungus were collected and analyzed for the first experiment. Five pooled samples of fungus were collected for the second experiment. Chemical analysis was performed on four of the five pooled samples of fungus. Dry weight and pH values represent results of all five samples.

Dry weight was determined by collecting the fungus on a glass fiber filter, washing with three 5-ml aliquots of distilled water, and filtering until most of the free water was removed. The damp glass-fiber filter plus fungus was then rolled up and placed into a 13 x 100 mm culture tube, frozen at -20°C, and lyophylized. Lyophylization was continued for four hours. Immediately after lyophilization, the rolled paper and fungus was removed from the culture tubes and weighed to the nearest tenth milligram on a Mettler analytical balance. After weighing, the filter paper and fungus were replaced in the culture tubes and the cotton plug was replaced. The culture tubes were then placed in 6-ounce prescription bottles and stored in a freezer.

pH Determination

pH of the medium was determined at harvest on a Beckman

Zeromatic pH meter. Readings were made daily after all of the samples had been collected. In the early portion of the growth curve when large numbers of flasks were involved, it was not possible to determine the pH of each separate culture. Instead, pooled filtrates from several flasks were used. Nine cultures were pooled in the first experiment, 15 in the second.

Fungus Analysis

Lipid Extraction

The total easily extractable lipid was determined by a modified chloroform/methanol method described by Bligh and Dyer (1959). Fungus and filter were ground in a Tenbrock glass homogenizer driven by a variable speed General Electric electric drill with a short piece of pressure tubing inserted into the top end of the pestle. The drill was plugged into a timer and into a variable transformer set at 80 volts. At full speed, the drill attained a speed of 900 rpm.

Fungus and filter were then homogenized for exactly eight minutes in nine ml of 2:1 methanol:chloroform. Then 2.7 ml of nonparticulate distilled water was added and homogenization continued for two minutes. After a total of ten minutes of homogenization, the contents of the homogenizer were filtered through a 4.25 cm diameter glass-fiber filter and the homogenizer was rinsed with the

filtrate, followed by three rinses of monophasic solvent 2:1:1 methanol: chloroform: water using 3.9 ml for each. Six and six-tenths ml of chloroform was then filtered through the homogenate collected on the filter. Lastly, 6.6 ml of nonparticulate distilled water was added directly to the centrifuge tube. The tube was then capped and the contents mixed. The tube and its contents were then placed in a freezer (-20°C) until all samples collected during one day had been homogenized. When enough samples were collected, they were centrifuged at 6000 rpm for five minutes (Sorval type SS-3 centrifuge, SS-34 rotor) to separate the aqueous layer from the chloroform layer. The aqueous phase was drawn off with a specially made volumetric pipette and stored in a clean test tube at -20°C for further analysis (see carbohydrate and apparent protein analysis). The pipette was an agar pipette with the tip drawn out to a fine point and slightly bent. With this pipette, the top aqueous layer could be drawn off completely with little loss of the lipid-containing chloroform layer.

The remaining chloroform layer was then poured into a preweighed, numbered aluminum dish. The centrifuge tube was rinsed with one ml of chloroform (the rinse added to the dish). The dish was then placed into a dust-free desiccator in a continuous flow of barium-oxide-dried and glass-wool-filtered air. When the solvent in the pans had evaporated, the pans were transferred to a second desiccator containing barium-oxide and left overnight. As a control, filters

without fungus material were ground and extracted, the resulting chloroform layer was evaporated in the same manner as the samples, the residue was weighed, and the mean of the blanks was subtracted from the sample weights. All weighings were done on a Mettler analytical balance.

Determination of the Relative Pigment Content

Glass filters with the adhering homogenized residue were placed in petri dishes at -20°C until needed. Each filter was moistened and allowed to equilibrate at room temperature until uniformly damp. From each filter, two 12 mm discs were punched out with a sharp cork borer. The reflectance of each disc was then determined on a reflectance apparatus (Brandt, 1967). The standard for 100% reflectance was a 12 mm disc of glass-fiber filter. The black-standard was made by layering partially purified pigment on a 12 mm glass-fiber filter. With the black standard in place, the reflectance apparatus was set at 0% reflectance.

The pigment was partially purified by heating a crude pigment preparation in 50% NaOH for 24 hours at 100°C. After 24 hours, the preparation was centrifuged, the NaOH was poured off, and the precipitate was acidified and then dried with acetone and ethyl ether. The purification process was repeated twice more. A total nitrogen analysis was performed on the remaining precipitate.

A standard reflectance curve was obtained by grinding a weighed amount of partially purified pigment with a clean glass-fiber filter in the same manner as the samples. The average reflectance of two discs from these knowns was used to determine the standard curve.

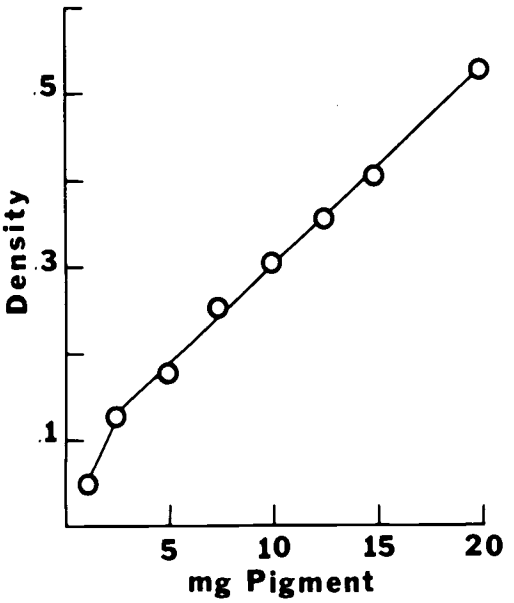

Figure 1. Standard reflectance of the partially purified pigment.

RNA Determination

Total RNA content of the fungus was determined by the orcinol method (Mejbaum 1939). The glass-fiber filter, plus the fungus residue on it, plus the two discs used for reflectance measurements were placed in a 13 x 100 mm culture tube with four ml of 4N NaOH

at 100°C for two hours.

In the first experiment the suspension was transferred to a polypropylene centrifuge tube, centrifuged at 8,000 rpm (Sorvall type SS-3 centrifuge, SS-34 rotor) for five minutes, and the clear supernatant poured off into a clean test tube. The precipitate was washed with four ml of distilled water. The tubes were recentrifuged and the second clear supernatant added to the first. Four ml of 0.1N HCl was added to each tube of supernatant and each was brought up to a 30 ml volume. The remaining precipitate was dried at 100°C.

In the second experiment, adapters were obtained which made it possible to centrifuge the culture tubes directly. The culture tubes and their contents were centrifuged at 5,000 rpm (Sorvall type SS-3 centrifuge, SS-34 rotor) for 20 minutes, each precipitate was rinsed with eight ml of distilled water, the tubes were recentrifuged, and the supernatant was pooled with the first. The precipitate remaining in the culture tube was dried at 100°C. The pooled nucleic acid fraction from the second experiment was diluted as in the first experiment.

An orcinol determination for RNA was performed on duplicate one ml aliquots of each sample. When duplicate results differed (by more than 5%), a third and fourth aliquot were run. Sodium ribonucleate (Nutritional Biochemicals Corporation) dissolved in 0.04N NaOH was used as a standard.

Two ml of distilled water was added to a test tube by means of

an Aupette followed by one ml of unknown, six ml of acid orcinol reagent (two ml of 100% $FeCl_3$ in 400 ml of concentrated HCl), and 0.4 ml of 6% ethanolic orcinol. The tube was stirred and placed in a boiling water bath for exactly 20 minutes. After 20 minutes, the tube was removed from the bath and placed in a cold water bath for five minutes. The absorbance of the standards and the unknowns were read on a Bausch and Lomb 340 Spectronic 20 spectrophotometer at 660 mμ.

DNA Determination

DNA content was determined by the method described by McIntire and Sproull (1957). A four ml aliquot of the nucleic acid fraction was added to a 13 x 100 mm culture tube with 0.2 ml of 1% protamine sulfate (Calbiochem), and placed in the refrigerator overnight. The next day, the cold samples were centrifuged for 10 minutes at 2,000 rpm. The supernatant was carefully poured off, the precipitate carefully washed with four ml of distilled water, the contents of the tube again centrifuged (2,000 rpm for five minutes), and the supernatant was poured off carefully. To the remaining precipitate, approximately three ml of 1% NaCl in 0.1N H_2SO_4 was added to the tube in one-ml aliquots. After each ml addition, the tube was shaken vigorously and the solution poured into a 10 ml graduated centrifuge tube. The graduated centrifuge tube was then

placed in a boiling water bath. After 20 minutes, the tube was removed and 10% NaCl in 0.1N H_2SO_4 was added to make a four ml volume. The contents of the centrifuge tube were then stirred well and were poured into a clean test tube. The test tube was capped with parafilm and kept in the refrigerator until the absorbance readings were made.

The absorbance was determined on a Beckman DB-G grating spectrophotometer at 268 mμ and 320 mμ. The 320 mμ values were subtracted from the 268 mμ values. The difference in the two values was assumed to be due to the DNA present in the solution. Salmon sperm DNA (Calbiochem) dissolved in 10% NaCl in 0.1N H_2SO_4 was used as a standard.

Chitin Determination

Chitin was determined by estimating the glucosamine content by the method of Elson and Morgan (1933). Glucosamine HCl (Pfanstiehl) was used as a standard. The dried precipitate from the nucleic acid extraction was sealed in a 13 x 100 mm culture tube after the addition of three ml of 6N HCl. The sealed tube was then placed into a 100^{o} oven for five hours. Then the tubes were removed from the oven and cooled. In the first experiment, the sealed tubes were broken open and the entire contents of each tube were transferred to a 50 ml polyproplene centrifuge tube. After centrifuging for 15

minutes at 8,000 rpm in a Sorvall type SS-3 centrifuge, SS-34 rotor, the clear supernatant was poured off into a clean test tube. An attempt was made not to pour off solid material, but this was very difficult because the solid had lost its stickiness. The precipitate was then washed with 10 ml of distilled water and was recentrifuged. The rinse was poured off and pooled with the first rinse.

In the second experiment, to eliminate the problem of separating the supernatant, the contents of the hydrolysis tubes were not transferred to a centrifuge tube. Instead, the clear liquid was first drawn off with a special pipette made of 10 mm outside diameter glass tubing drawn out to a very narrow tip. The remaining contents of the hydrolysis tubes were then transferred to 13 x 100 mm culture tubes. Four ml of distilled water was used to rinse the broken tubes then added to the culture tube also, thoroughly mixed, and then centrifuged for 15 minutes at 5,000 rpm (Sorvall type SS-3, SS-34 rotor). The clear supernatant was drawn off with the special pipette. A second four-ml distilled water rinse was added, the mixture was recentrifuged, and all rinses collected and pooled in the same clean test tube.

Pooled supernatant from each sample in both the first and second experiment was brought to 25 ml. Two and five-tenths ml of this fraction from each sample was pipetted into clean test tubes to be used for protein and carbohydrate determination. The remainder of the acid hydrolysis fraction was put into a round-bottom flask and

evaporated to dryness on a rotating vacuum evaporator in a water bath. The residue was dissolved in five ml of distilled water. Two one-ml aliquots were drawn off into separate test tubes (in the first experiment, one one-ml and one 1/2-ml sample were drawn off instead). The glucosamine present was determined by the method of Elson and Morgan (1933) with one modification. Instead of using one ml of acetylacetone, two ml were used. This change was necessary because an unknown sample with an internal standard did not produce the expected amount of color.

Total Carbohydrate Determination

The total carbohydrate content was determined by the anthrone method of Morris (1948).

The aqueous layer of the lipid extraction procedure was evaporated to dryness and the residue dissolved in 20 ml of 0.4N NaOH. Two ml or 1/10 of this fraction was put into a test tube with 1/10 of the other fractions (nucleic acid and acid hydrolysis fraction).

The total carbohydrate of the sample was determined by the anthrone reaction on an aliquot of the pooled fractions. In the first experiment, 0.2 ml of the pooled fraction was added to a 13 x 100 mm culture tube with 0.8 ml of water. The culture tube was inserted in a hole drilled in a block of polystyrene foam to conserve the heat of the subsequent reaction and two ml of anthrone reagent (two grams

anthrone in one liter of 95% H_2SO_4) was added to the tube. The tube was stirred on a Cyclomixer while still in the foam block and the reaction allowed to continue about two minutes. The absorbance was read on a Bausch and Lomb model 340 Spectronic 20 spectrophotometer at 540 mμ. Glucose was used as a standard. In the second experiment, 0.1 ml of unknown was used instead of 0.2 ml.

Total Apparent Protein Determination

The total apparent protein was determined by the method of Lowry et al. (1951).

A protein determination was carried out on an aliquot of the pooled sample described above. Three-tenths ml of unknown and 0.3 ml water were added to a 13 x 113 ml culture tube. With an Auppette, three ml of alkaline copper solution was added to each tube. The tubes were stirred on a Cyclomixer and allowed to remain at room temperature for 10 minutes. After 10 minutes, 0.3 ml of phenol reagent was added to each tube and the tube was shaken immediately. After 30 minutes at room temperature, the absorbance of the reaction solution was determined on a Bausch and Lomb model 340 spectrophotometer at 700 mμ. Unknowns were run in duplicate. For a standard, recrystallized, lyophilized, bovine serum albumin was used.

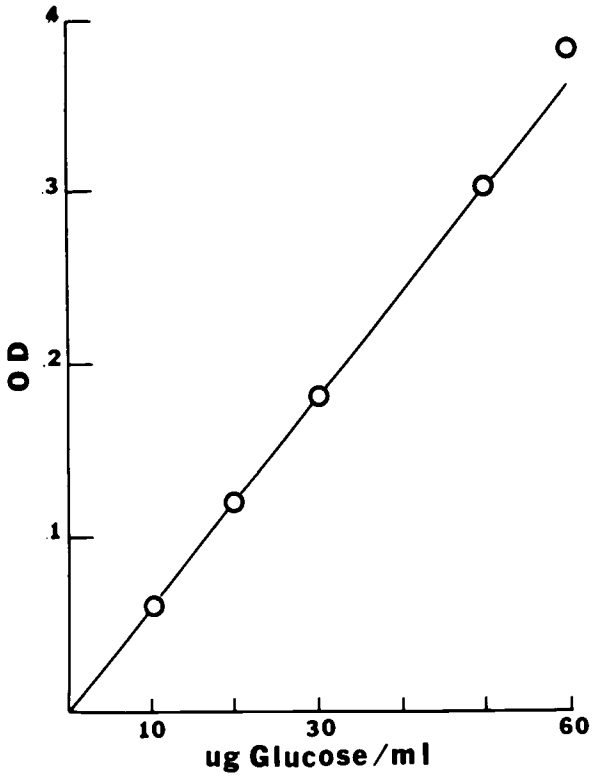

Figure 2. Standard curve for glucose reacted with anthrone reagent.

RESULTS

Total Percent Lipid

The percent lipid content of the dark-reared cultures in Experiment 1 increased rapidly after the first day and, except for the tenth day, remained higher than the lipid content of the UV-reared cultures (Figure 3, Experiment 1). The lipid content of the dark-reared cultures of Experiment 2 was generally about double the lipid content of the UV-reared cultures (Figure 3).

No lipid analysis was performed on one-day-old cultures in Experiment 2; therefore, the rapid increase in lipid content which occurred between days 1 and 2 in Experiment 1 could have not have been observed in Experiment 2 although it may have occurred.

Relative Pigment Content

In Experiment 1, pigment was detected as early as the second day. There was then a rapid increase in pigment content until a maximum was reached on the fifth day. The relative pigment content decreased after the fifth day and then increased again by the tenth day (Figure 4, Experiment 1).

In Experiment 2, pigment was not detected until the third day. The increase in pigment content was not as rapid nor as great as in Experiment 1 (Figure 4, Experiment 2).

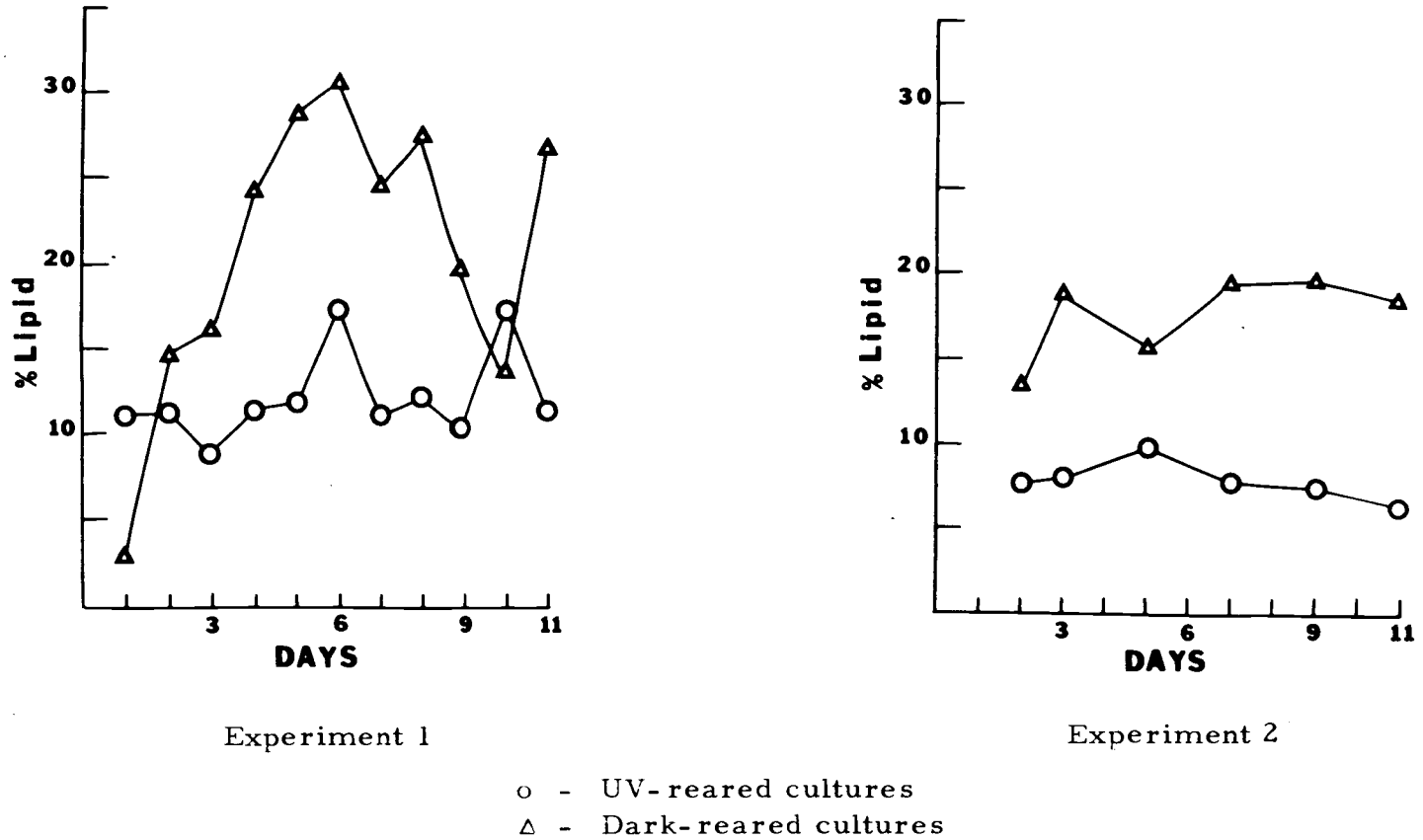

Figure 3. Effect of UV radiation on lipid content of Verticillium albo-atrum. Dark-reared cultures harvested on days 2, 6, 7, 8, 9, and 11 of Experiment 1, and days 2, 3, 7, 9, and 11 of Experiment 2 contained significantly more lipid at the 5% level according to the U-test of Mann and Whitney (1947) than did the UV-reared cultures. In Experiment 1, most points represent the mean lipid content of three independent pools of fungus cells. In Experiment 2, most points represent the mean lipid content of four independent pools of fungus cells.

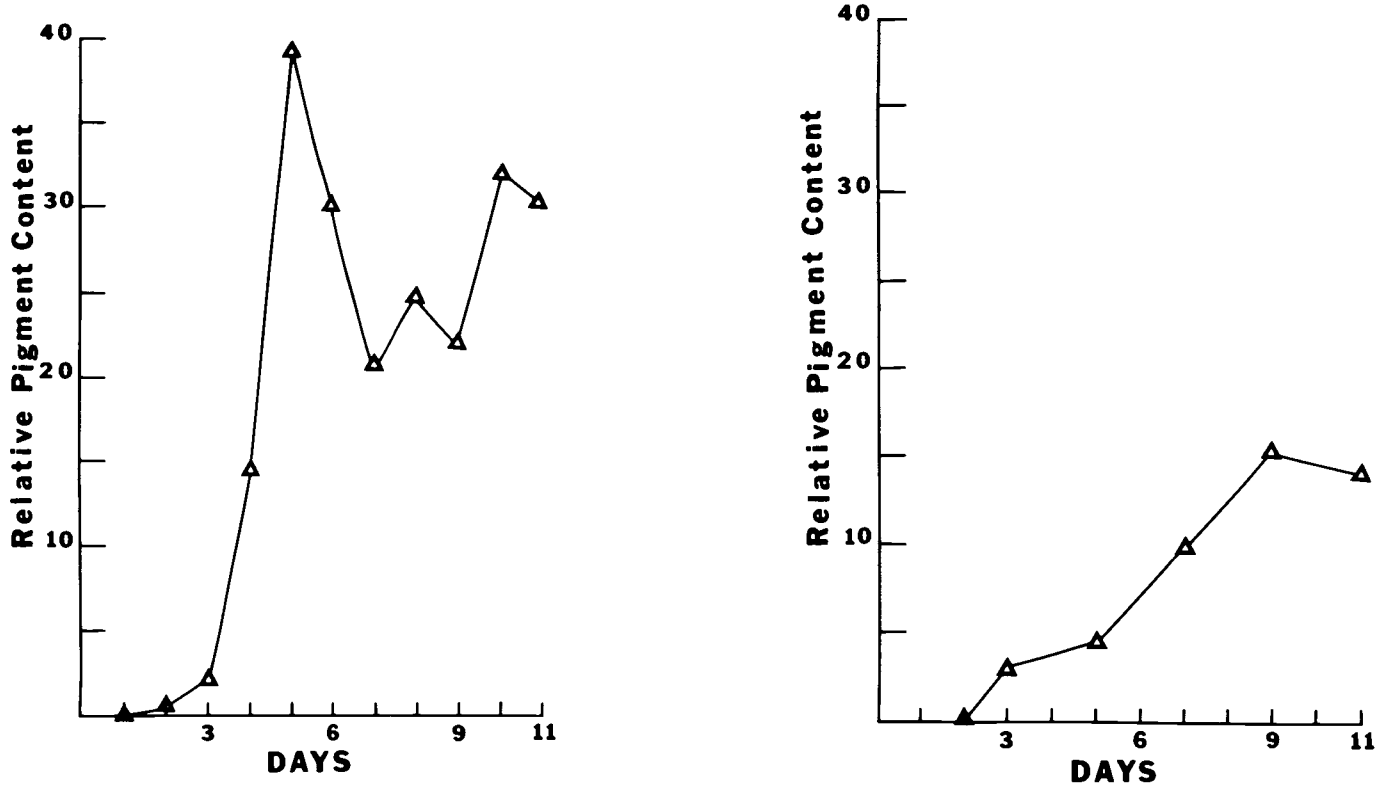

Figure 4. The relative pigment content (apparent mg pigment/dry weight x 100) of dark-reared cultures of Verticillium albo-atrum. Relative pigment content also represents microsclerotia content. UV-reared cultures did not produce pigment or microsclerotia. In Experiment 1, each point represents the mean relative pigment content of three independent pools of fungus cells. In Experiment 2, each point represents the mean relative pigment content of four independent pools of fungus cells.

Percent Apparent Protein

There was, at first, a decrease in percent apparent protein in UV-reared and dark-reared cultures of Experiment 1. The percent apparent protein of UV-reared cultures remained between 17-29% throughout the remainder of the growth period. The percent apparent protein of the dark-reared cultures of Experiment 1 decreased, except on the fourth and fifth days, and reached a low of 8% by the eleventh day (Figure 5, Experiment 1).

In Experiment 2, the apparent protein content of UV-reared cultures remained between 20-25% throughout the entire growth period. The apparent protein content of the dark-reared cultures decreased steadily from 21% and reached a low of approximately 8% by the eleventh day (Figure 5, Experiment 2).

Percent RNA

The RNA content of the UV-reared cultures of Experiment 1 tended to remain higher than the RNA content of the dark-reared cultures. There was a progressive decrease in RNA content in both UV-reared and dark-reared cultures of Experiment 1 (Figure 6, Experiment 1).

The percent RNA in the UV-reared cultures of Experiment 2 remained relatively constant throughout the growth period. The RNA content of the dark-reared cultures remained lower throughout the

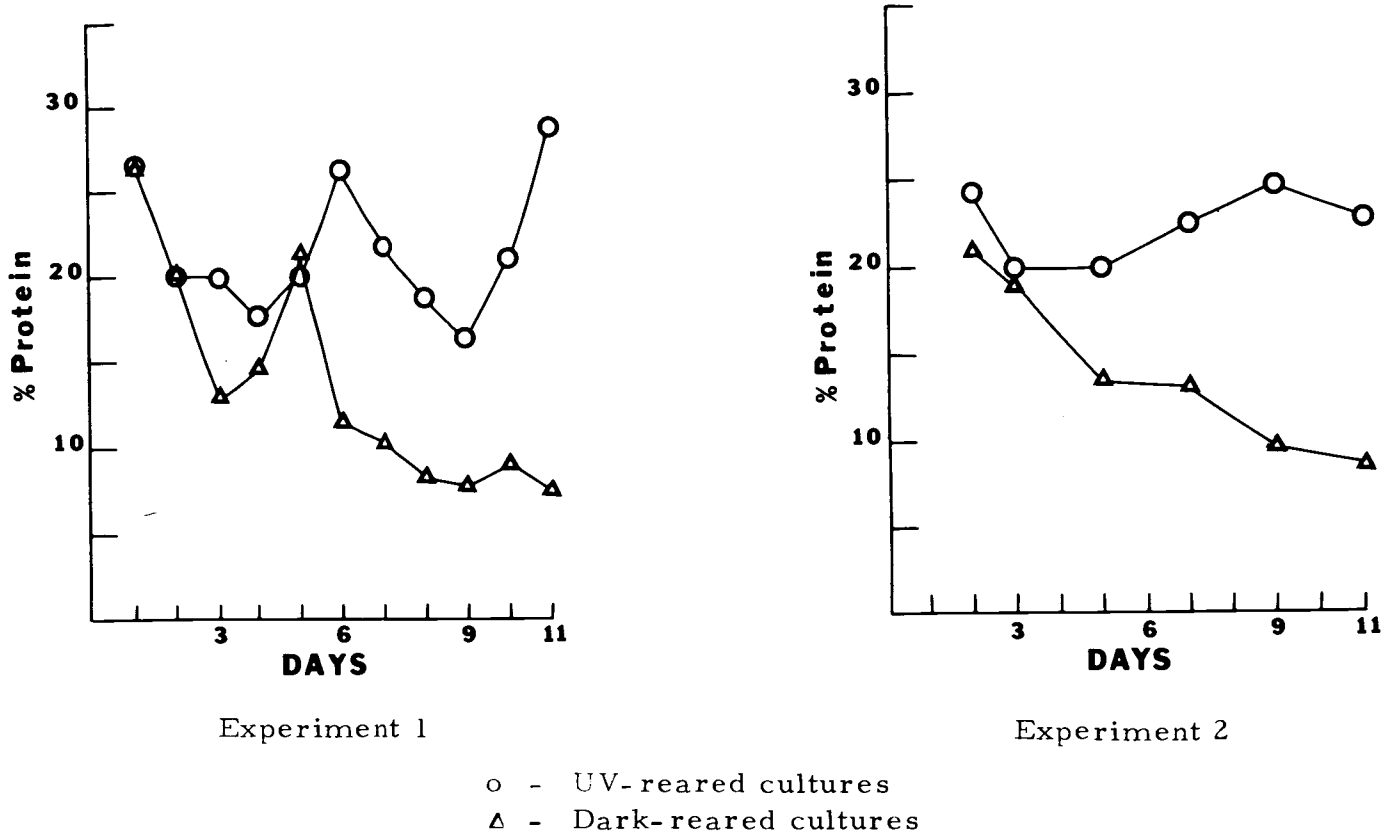

Figure 5. Effects of UV radiation on apparent protein in Verticillium albo-atrum. UV-reared cultures harvested on days 4, 6, 7, 8, 9, 10, and 11 of Experiment 1, and days 5, 7, 9, and 11 of Experiment 2 contained significantly more apparent protein than dark-reared cultures at the 5% level according to the U-test of Mann and Whitney (1947). In Experiment 1, each point represents the mean of three independent pools of fungus cells. In Experiment 2, each point represents the mean of four independent pools of fungus cells.

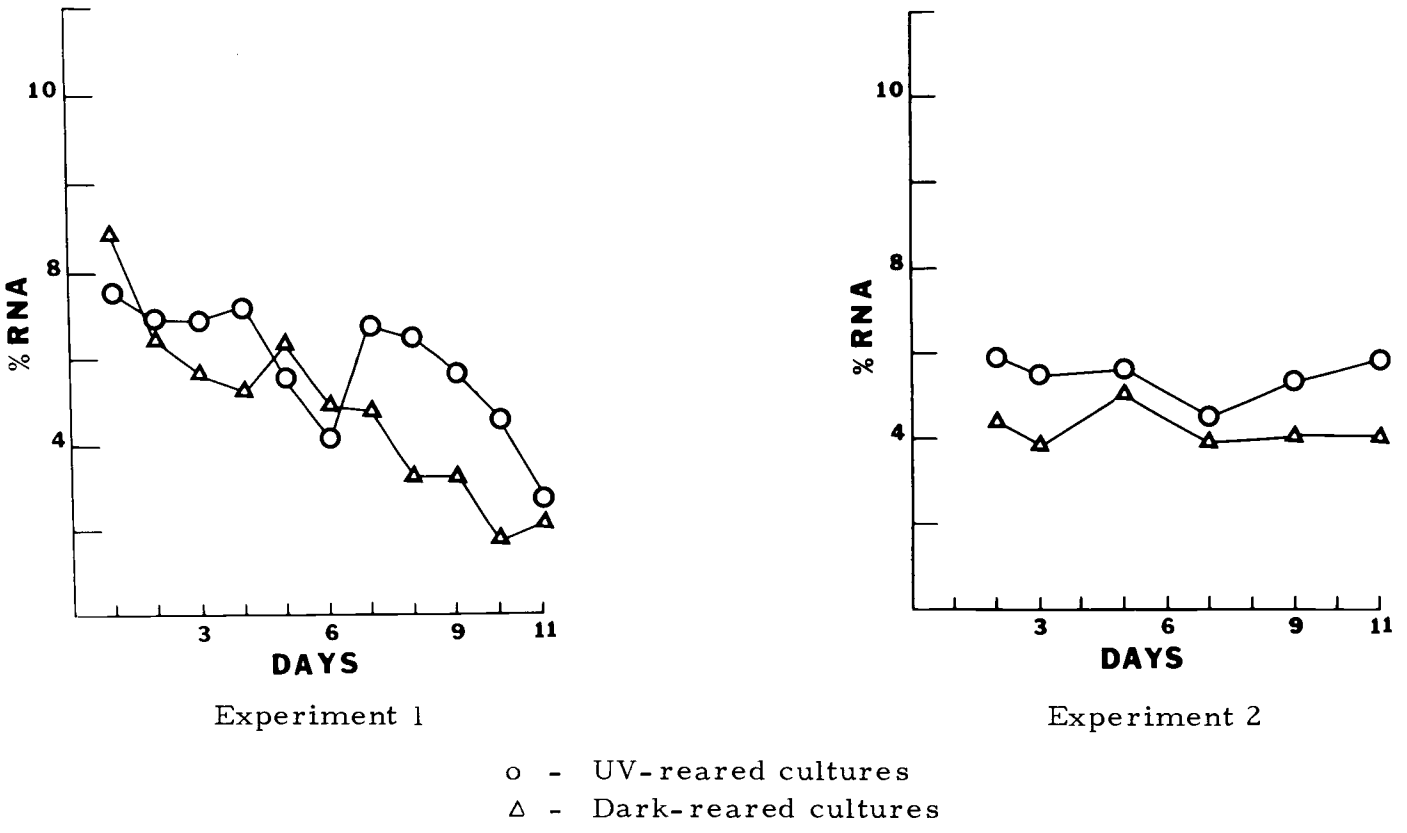

Figure 6. Effect of UV radiation on RNA content of Verticillium albo-atrum. UV-reared cultures harvested on days 4 and 8 in Experiment 1, and days 2, 7, 9, and 11 in Experiment 2 contained significantly more RNA at the 5% level than did the dark-reared cultures according to the U-test of Mann and Whitney (1947). In Experiment 1, each point represents the mean of three independent pools of fungus cells. In Experiment 2, each point represents the mean of four independent pools of fungus cells.

growth period (Figure 6, Experiment 2).

The only similarity in RNA content data of Experiment 1 and 2 is the higher RNA content present in UV-reared cultures.

Percent DNA

With few exceptions, the DNA content of the UV-reared and dark-reared cultures of both Experiment 1 and 2 remained at a very low level. No consistent trend in DNA content with age or treatment of the cultures was observed (Figure 7).

Percent Chitin

The chitin content of both UV-reared and dark-reared cultures of Experiment 1 and 2 generally remained at a low level. No consistent trend in chitin content of cultures was made apparent by age or treatment of the cultures (Figure 8).

Percent Carbohydrate

The carbohydrate content of dark-reared and UV-reared cultures of Experiment 1 did not follow any discernible pattern except that the dark-reared cultures tended to contain more carbohydrate than the UV-reared cultures (Figure 9, Experiment 1).

Unlike Experiment 1, the carbohydrate content of dark-reared and UV-reared cultures of Experiment 2 did seem to follow a pattern.

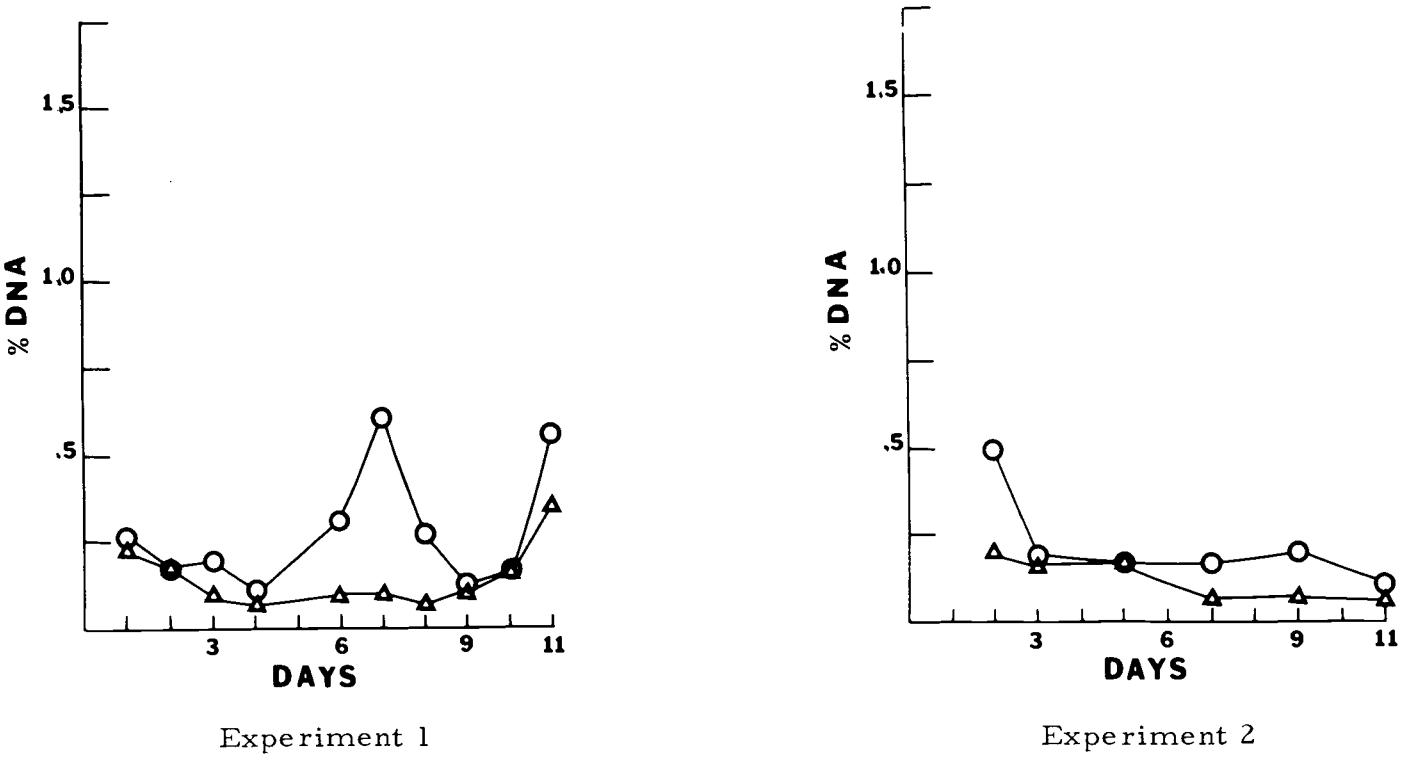

Figure 7. Effect of UV radiation on DNA content of Verticillium albo-atrum. UV-reared cultures harvested on days 3, 6, 7, and 8 in Experiment 1, and days 2, 9, and 11 in Experiment 2 contained significantly more DNA at the 5% level than the dark-reared cultures according to the U-test of Mann and Whitney (1947). In Experiment 1, most points represent the mean DNA content of three independent pools of fungus cells. In Experiment 2, most points represent the mean DNA content of four independent pools of fungus cells.

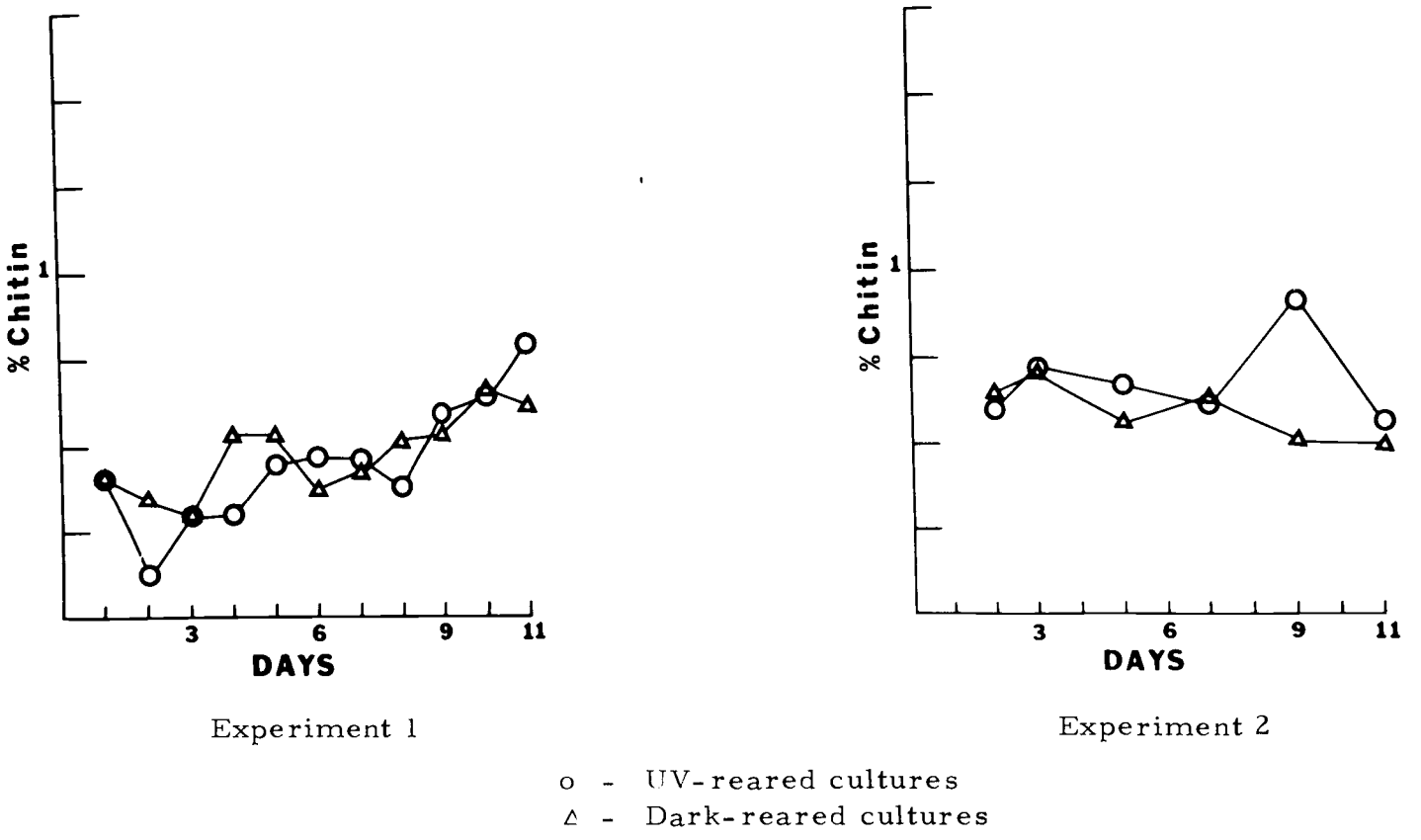

Figure 8. Effects of UV radiation on chitin content of Verticillium albo-atrum. Only UV-reared cultures on day 9 in Experiment 2 contained significantly more chitin at the 5.. level than did the dark-reared cultures according to the U-test of Mann and Whitney (1947). In Experiment 1, each point represents the mean chitin content of three independent pools of fungus cells. In Experiment 2, each point represents the mean chitin content of four independent pools of fungus cells.

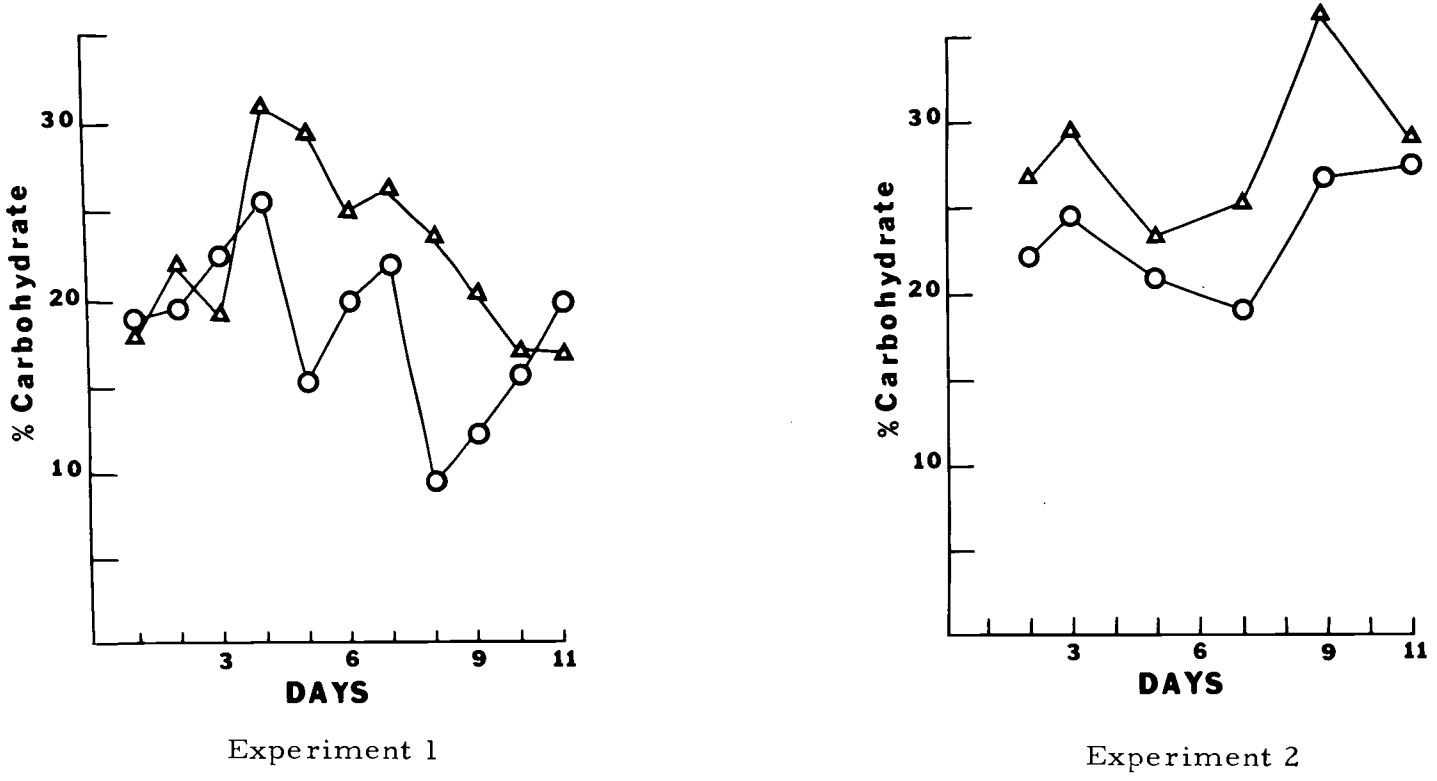

Figure 9. Effect of UV radiation on carbohydrate content of Verticillium albo-atrum. Dark-reared cultures harvested on days 6, 7, and 8 in Experiment 1, and days 5, 7, and 9 in Experiment 2 contained significantly more carbohydrate at the 5% level than did UV-reared cultures according to the U-test of Mann and Whitney (1947). In Experiment 1, most points represent the mean carbohydrate content of three independent pools of fungus cells. In Experiment 2, most points represent the mean carbohydrate content of four independent pools of fungus cells.

The pattern followed by both dark-reared and UV-reared cultures was that the carbohydrate content reached a maximum on the third day and then declined. After the seventh day, there was a second increase in carbohydrate content.

As in Experiment 1, the dark-reared cultures tended to contain more carbohydrate than did the UV-reared cultures (Figure 9, Experiment 2).

Dry Weight

The dry weight of dark-reared cultures in Experiment 1 and 2 increased at a much faster rate than did the dry weight of UV-reared cultures.

By the eleventh day, the dark-reared cultures of Experiment 1 contained three times more dry weight of fungus material than the UV-reared cultures. By the eleventh day, the dark-reared cultures of Experiment 2 contained four times more dry fungus material than UV-reared cultures.

In the first experiment, the log phase of the Verticillium growth in the dark lasted 48 hours. The log phase, if any, of the Verticillium growth in ultra-violet radiation in the first experiment lasted less than 24 hours. In Experiment 2, neither the dark-reared cultures nor the UV-reared cultures displayed log phases of growth. If there was any log phase, it must have occurred prior to the first

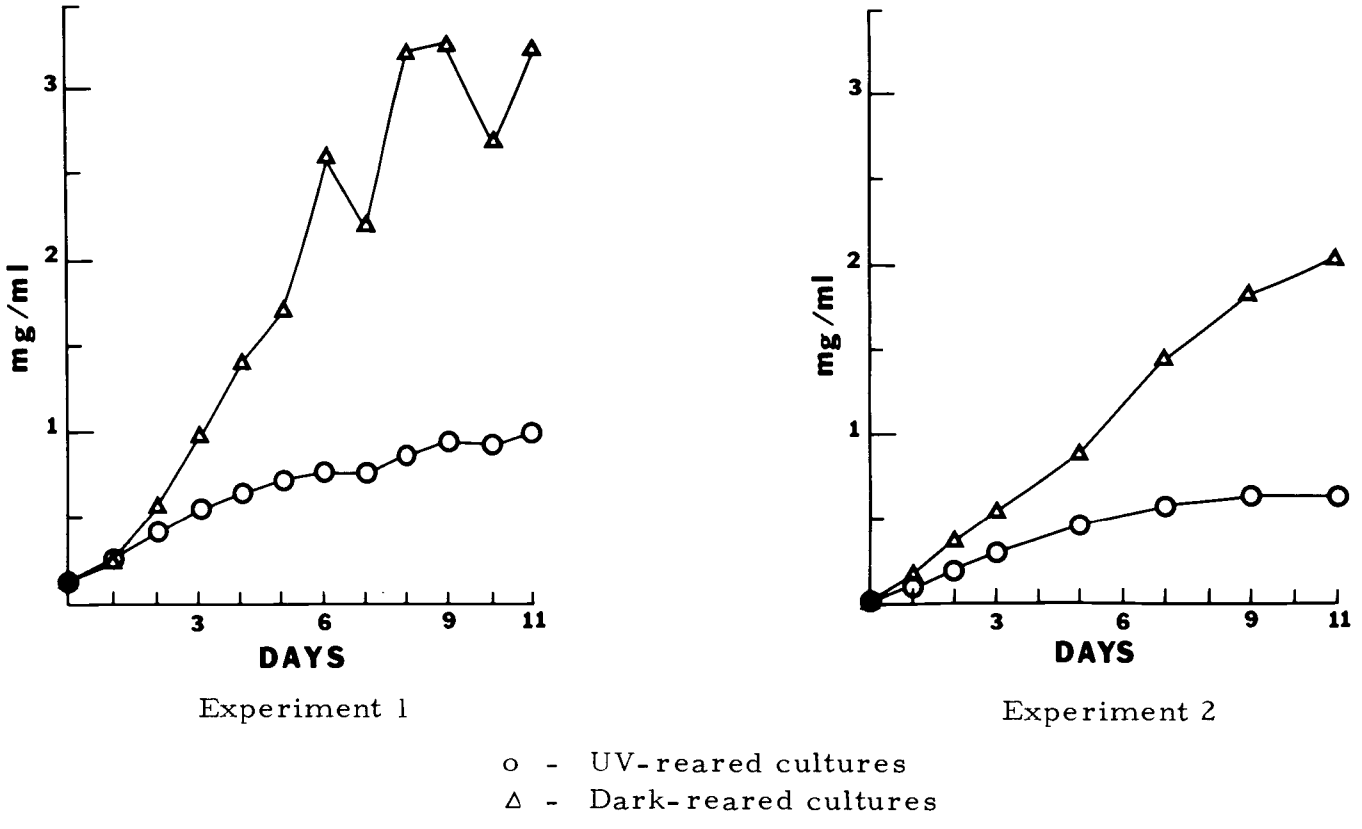

Figure 10. Effect of UV radiation on the dry weight of Verticillium albo-atrum cultures. In Experiment 1, each point represents the mean dry weight of three pools of fungus cells. In Experiment 2, each point represents the mean dry weight of five pools of fungus cells.

harvest, i.e., during the first 24 hours.

pH

The pH of the medium of dark-reared cultures in both experiments increased progressively throughout the gorwth period. The pH of the UV-reared cultures in Experiment 1 remained approximately the same throughout the experiment, but in Experiment 2 there was a progressive decrease in pH of the medium.

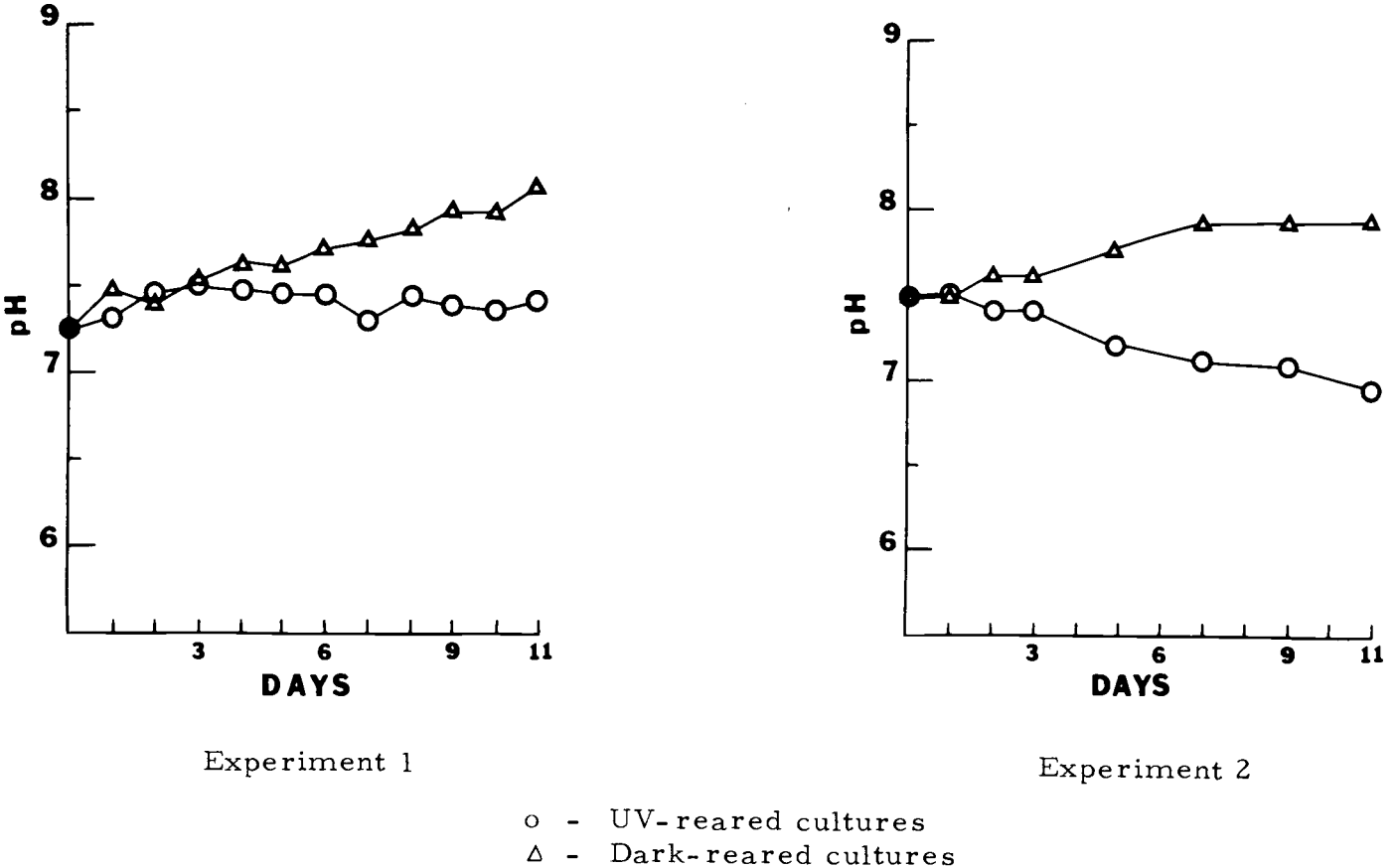

Figure 11. Effect of UV radiation on pH changes in culture medium of Verticillium albo-atrum. In Experiment 1, each point represents the mean pH of three pools of culture media. In Experiment 2, each point represents the mean pH of five pools of culture media.

DISCUSSION

Total Percent Lipid

The substantial reduction in percent lipid content of Verticillium exposed to near-UV radiation during growth (Figure 3) constitutes one of the most interesting findings of this study. Since near-UV radiation supresses pigment synthesis and microsclerotia development in Verticillium, a connection between lipid metabolism, pigment synthesis, and microsclerotia development is suggested. The present study provides some additional evidence of such a connection: dark-reared cultures in the first experiment contained 30-100% more pigment than did dark-reared cultures in the second experiment; they also contained 25-50% more lipid than did dark-reared cultures in the second experiment.

In at least one other organism there seems to be a connection between lipid metabolism and pigment synthesis. Chet et al (1967) have found that pigment-containing sclerotial cell walls of Sclerotium rolfsii contained twice as much lipid as did hyphal walls of the same fungus.

The exploration of the possible connection between lipid metabolism, pigment synthesis, and microsclerotia development in Verticillium must await further research. However, a hypothesis about a possible connection may be in order at this time.

Brandt (1965) and MacMillan and Brandt (1966) have suggested that melanin synthesis itself or some event closely related to it may play a causal role in microsclerotia development. Brandt (1965) and MacMillan and Brandt (1966) indicate that phenols of some sort may be precursors of Verticillium melanin. If this is true, and if those phenolic precursors of melanin are formed via the polyacetate pathway, as appears to be common in fungi (Bentley, 1962), then an agent which interferred with the polyacetate pathway might interfere with both lipid metabolism and the biosynthesis of at least some phenols. The interference with phenol biosynthesis could, in turn, result in decreased melanin synthesis and decreased microsclerotia development as well.

Near-UV radiation could be such an agent since it does inhibit production of microsclerotia, synthesis of melanin, and synthesis of lipid in Verticillium.

Relative Pigment Content

It is important to quantitatively determine the pigment content of Verticillium if only because of the bulk that it may represent. Some organisms which have been analyzed for melanin content in cell walls are Rhizoctonia solni, 8.5%; Cladosporium sp., 12.8% (Potgeiter and Alexander, 1966); and Aspergillus nidulans, 4.9% (Kuo and Alexander, 1967). In the case of Verticillium, it becomes even

more important to attempt a pigment analysis because of the suspected role played by pigment biosynthesis in microsclerotia development. The melanin content of Verticillium, moreover, provides an approximate measure of the number of microsclerotia since in the isolate used in this study the pigment occurs only in the microsclerotia.

The pigment found in Verticillium may also play an important role in its survival and ecology in the soil. Evidence has been given that suggests that surface localized melanins or related compounds have great ecological significance (Lockwood, 1960; Kuo and Alexander, 1967; Potgeiter and Alexander, 1966; Bloomfield and Alexander, 1967). The presence of melanins inhibits the lytic enzymes β-(1$\rightarrow$3) glucanase and chitinase (Kuo and Alexander, 1967). This inhibition has been correlated with the ability of certain organisms to survive long periods in the soil.

In spite of the biological importance of melanins, no adequate method for their quantitative analysis is yet available. The method of Nicholous et al. (1964) seems to be the most widely used, but is long and tedious. Since melanin is not "a single chemical compound of definite composition" (Swan, 1963), the validity of the Nicholous method is somewhat uncertain.

The day to day increase in pigment cannot be easily reported in meaningful terms with the "eyeball" method which is also in common use. The relative pigment content data (apparent mg pigment/mg dry

weight x 100) obtained in this work gives one access to objective comparison of each culture to the same standard. With this data, one can rapidly determine if one culture contains more or less pigment (microsclerotia) per mg dry weight than does another culture.

Brandt (1967), who used the same reflectance apparatus as that used in the present study, has shown a direct correlation between number of microsclerotia and the absorbance of microcultures of Verticillium on glass fiber discs.

Percent Apparent Protein

Other fungi grown under laboratory conditions have shown a decrease in percent protein as cultures aged. Gottlieb and Van Etten (1964) observed a decrease in percent protein with time in Penicillium atrovenetum; Singh (1966), working with Claviceps purpurea, and Dorn and Rivera (1960), working with Aspergillus nidulans, obtained similar results. Dorn and Rivera (1960), however, did get an increase in percent protein in cultures of Aspergillus nidulans which had a high phosphate content. In all cases, where there was a decrease in percent protein, the decrease always seemed to begin very shortly after the fungus would be expected to have completed its true logrithmic phase of growth. Generally, the decrease continued throughout the remainder of the growth curve.

In this study of Verticillium, the presence of Folin reagent

reducing compounds was designated as apparent protein. The term "apparent protein" was chosen because it is known that other compounds besides protein will reduce the Folin reagent. Some compounds known to reduce the Folin reagent include phenols. It is possible that phenols could be present in Verticillium cytoplasm, especially during melanin biosynthesis.

The dark-reared cultures of Verticillium showed a decrease in apparent percent protein with increasing age. The decrease, as in the case of the above mentioned fungi, also began shortly after one would have expected the log phase in the growth curve to have been completed. The decrease in apparent percent protein could be attributed to the reduced metabolic activity of the microsclerotia of Verticillium. Since microsclerotia appear to be dormant structures, they would be expected to contain fewer enzymes and thus possibly decreased apparent protein. A second possibility is that there were larger amounts of phenolic precursors present in the cytoplasm of Verticillium and, as the pigment was formed, the phenols were used up and this was reflected in decreased percent apparent protein.

The UV-reared cultures showed no decrease in the percent apparent protein with increasing age. This could have been due to the maintenance of a high enzyme content or to the presence of the high concentrations of phenolic precursors. The higher enzyme level seems plausible since the UV-reared cultures also maintained a

higher RNA level.

Total Percent RNA

RNA analysis of several fungi has shown that RNA content can vary considerably with age of the fungus. Percent RNA from some studies are: Penicillium atrovenetum, 1-3% (Gottlieb and Van Etten, 1964); Neorospora crassa, 6.9-9.1% (Lester, 1965); Claviceps purpurea, 2.6-6.8% (Singh, 1966); Candida albicans, 1.8-2.4% (Sujata, 1966).

Messenger RNA is essential for protein synthesis and most of the RNA present in the cell is of the m-RNA type. Therefore, one might expect to see some correlation between the protein content of an organism and its RNA content. Such a correlation has been observed in Penicillium atrovenetum (Gottlieb and Van Etten, 1964) and Claviceps purpurea (Singh, 1966). In both studies, it was observed that with increasing age there was a decrease in RNA content and a subsequent decrease in the protein content. In the UV-reared cultures of Verticillium, where the apparent protein content remained at high level (Figure 5, p. 23), there was a higher RNA level also (Figure 6, p. 24). In the dark-reared cultures where the protein content decreased in progressively older cultures, significantly less RNA was present compared to UV-reared cultures. No direct relation between RNA content and microsclerotia

development was apparent.

Percent of DNA Content

The percent DNA in fungi seems to be generally quite low. Gottlieb (1964) obtained 0.5-0.75% DNA in Penicillium atrovenetum. Singh (1966), working with Claviceps purpurea, obtained a constant 0.3% DNA. Lester (1965) obtained 0.33-0.34% with Neurospora crassa, and Sujata (1966), working with Candida albicans, obtained an unusually high 1.0-1.2% DNA. The percent DNA in Verticillium, however, appeared to be generally lower than found in the above fungi. This suggests that Verticillium has an unusually low DNA content or that a special technique for measuring DNA in Verticillium will have to be developed. The DNA content present in the dark-reared cultures was not significantly different from the DNA content of the UV-reared cultures. Therefore, no direct relation between DNA content and morphogenesis of microsclerotia was apparent.

Total Percent Chitin

The amount of hexosamine in sclerotia and hypal walls of Sclerotium rolfsii was measured by Chet et al. (1967). The percent of hexosamine in the hypae was double that in the sclerotia. There was no difference in the hexosamine content of the light-reared or dark-reared cultures. Gottlieb and Van Etten (1964) with P.

atrovenetum, and Singh (1966) with Claviceps purpurea, did not observe any significant changes in chitin content with aging. There were also no changes in chitin content observed in Verticillium. It appears that chitin production in Verticillium is not correlated with aging of the cultures, with melanin synthesis, or with the production of microsclerotia.

Percent Total Carbohydrate

Carbohydrates are a major biochemical component of fungi. Various fungi have carbohydrate contents varying from 32-60% (Cochrane, 1958). Gottlieb and Van Etten (1964) obtained carbohydrate content ranging from 20-45% of the total dry weight of Penicillium atrovenetum. Borrow et al. (1961), working with Gibberella fujikuroi, obtained 12-32% carbohydrate, depending on the age of the fungus analyzed. In his work with Claviceps purpurea, Singh (1966) obtained values varying from 20-32% of the total dry weight. If the above values could be considered a criterion for normal carbohydrate content then one could say that Verticillium had carbohydrate content within the normal range. This study has shown that dark-reared, microsclerotia-producing cultures of Verticillium contain more carbohydrate at least part of the time than do UV-reared cultures.

Dry Weight

The much lower dry weight produced by UV-reared cultures was unexpected because another Verticillium isolate, isolate H-13, produced roughly the same total dry weights of dark-reared cultures and in UV-reared cultures (Brandt, 1963). Experiments with isolate H-13 showed that dry weights of both dark-reared and UV-reared cultures reached maxima after ten days (Brandt, 1963). A reason for the difference in reaction of the two isolates was sought. In reviewing the lighting conditions used with H-13, it was found that the intensity of the light was 1/2 to 3/4 as great as the light used in this study. This is possibly the explanation for the differences obtained with the two isolates. It has been found that higher intensity light will reduce the dry weight in yeast (Ehrenburg, 1966).

The growth curve of the dark-reared cultures in the first experiment was substantially less smooth than the growth curve of the dark-reared cultures in the second experiment. Some of the variability that was not overcome by sufficient sampling in the first experiment could be attributed to the filter sterilization technique used. It is now suspected that the detergents present in the Millipore filters may reduce microsclerotia production. Detergents have been shown to reduce microsclerotia formation in Verticillium isolate H-13 (Brandt, 1967b). The filter sterilization technique used in the

first experiment made it possible for the detergent content to vary considerably from one culture to the next. In the second experiment, the filter sterilization technique would have tended to distribute the detergent more evenly between the flasks.

pH of the Medium

Changes in the pH of a fungus culture medium can be attributed to the selective uptake of one or more ions or to the release of one or more metabolites into the medium. Most generally the above causes do not act alone. The final pH of the medium is a result of a combination of the two effects.

Of the many metabolites produced by fungi, the production of an acid is quite common. The rising pH of the dark-reared cultures with age of the cultures suggests that there was no acid or not enough acid produced by the fungus to counteract the pH change due to the uptake of anions. The light-reared cultures, however, seemed to produce larger amounts of acid or there was less anion removal from the culture medium. Therefore, there was a decrease in pH with increasing age of the cultures.

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