Action Spectrum of the Photoinduced Sexual Stage in the Fungus Nectria haematococca Berk. and Br. var. cucurbitae (Snyder and Hansen) Dingley1,2

C. R. CURTIS
Department of Botany, University of Maryland, College Park, Maryland 20742

ABSTRACT

An action spectrum was determined for the photoinduced formation of perithecia in a homothallic strain of Nectria haematococca Berk. and Br. var. cucurbitae (Snyder and Hansen) Dingley. Dose-response curves for perithecial formation were obtained from 340 to 510 nanometers at 10-nanometer intervals. Radiation longer than 510 nanometers was not effective for inducing perithecial formation. The action spectrum indicated peaks of activity near 360, 440, and 480 nanometers with shoulders near 420 and 460 nanometers. Minima occurred near 350 nanometers, 390 to 410 nanometers, and 470 nanometers. The general shape of this action spectrum appears to be similar to those obtained for many different near ultraviolet-blue-sensitive organisms in which a flavoprotein molecule was postulated to be the photoreceptor.

Previous research has shown that a photorequirement exists for proper development of asexual and sexual reproductive structures in several closely related strains of the fungus Nectria haematococca Berk. and Br. var. cucurbitae (Snyder and Hansen) Dingley (2, 8, 9, 25, 33). These strains are heterothallic or homothallic and have a common imperfect stage of Fusarium solani (Mart.) App. and Wr. emend. Synd. and Hans. Although Snyder and Hansen (25) reported that light was necessary for development of the asexual (macroconidia) stage and sexual (perithecia) stage in many Fusarium species, Baker (2) found that light intensity in particular had a marked effect on the production of the sexual (perithecia) stage. Of three intensities used, the optimal intensity for stimulating the production of perithecia in heterothallic strains was 190 to 195 ft-c.

In a more comprehensive study of the light requirement, Curtis (8) found a broad optimal range of intensity for the production of perithecia from 50 to 250 ft-c. Darkness or higher intensities of irradiation greatly inhibited the formation of perithecial primordia and perithecia. In addition, a region of spectral sensitivity in the near UV-blue portion of the spectrum was found for two heterothallic strains and a homothallic strain.

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MATERIALS AND METHODS

Organism. The perithecial form was recognized as Nectria haematococca Berk and Br. var. cucurbitae (Snyder and Hansen) n. comb (11). A perfect stage synonym is Hypomyces solani Rke and Berth. emend Snyder and Hansen var. cucurbitae (26). The name N. haematococca var. cucurbitae will be followed in this paper.

The fungus used in this research was a homothallic isolate obtained from Dr. W. C. Snyder, Berkeley, California and was originally designated S29. This isolate was selected for study because it readily forms red-orange perithecia in culture and does not require the additional step of fertilization by an opposite compatibility strain in order to produce perithecia.

Growth Medium and Culturing. The fungus was grown on a medium containing 30% (v/v) V-8 juice (Campbell Soup Co.), 3 g of CaCO3, 15 g of agar (BBL), and distilled water to one liter. After autoclaving the medium, 15 ml aliquots were aseptically transferred into 100-×15-mm plastic Petri dishes (Optilux. Falcon Plastics, Oxnard, Calif.). Following solidification of the medium, the Petri dishes were inverted and stored at 4°C until used for starting colonies of the organism. A colony was initiated by placing a single macroconidium on the medium adjacent to the side wall of the Petri dish. Macroconidia for these initial colonies were obtained from stock cultures of the organisms maintained on 1.5% (w/v) agar. After seeding the medium, the Petri dish lid and bottom were taped together at two points to avoid accidental opening and contamination during the incubation periods in darkness and also during the irradiation treatment.

The colonies were allowed to grow for 10 days at 24°C in darkness. Approximately half of the agar surface was covered by mycelia after this time. It was then necessary to prepare the colonies for mounting on an optical bench prior to irradiation. This was accomplished under a dim biological red safelight provided by a red fluorescent lamp (G.E. F40R) which has a lower cutoff point near 575 nm and maximum emission near 640 nm. It was previously determined that the organism was not sensitive to wavelengths longer than 510 nm (8). All manipulations required during the irradiation phase of the experiments and subsequent incubation period in darkness were performed in a specially constructed dark room that contained the monochromator and irradiation measuring equipment.

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**Instrumentation.** To produce the required monochromatic radiation, the arc from a 150 w xenon lamp was focused on the entrance slit of a quartz optics, 500 mm, diffraction grating monochromator (Bausch and Lomb, Rochester, N.Y.). A series of glass filters (Corning Glass Works, Corning, N.Y.) was employed to absorb stray light and eliminate the second order spectrum. A 10 nm band width was set for all experiments. The diffraction grating for the monochromator was blazed at 400 nm with 600 grooves per mm. When it was necessary to vary the intensity of irradiation, suitable neutral density filters were positioned in a filter holder in front of the monochromator entrance collective lens. An optical bench was used to insure accurate positioning of (a) filters, (b) a large lens holder for positioning culture plates, and (c) a thermopile for irradiation measurements.

The irradiation intensity was accurately and conveniently measured with a calibrated, linear, CaF₂, window, 12-junction bismuth-silver thermopile (Eppley Laboratory, Inc., Newport, R.I.). The thermopile was coupled to a nanovoltmeter (Model 148, Keithley Instruments, Inc. Cleveland, Ohio) and strip chart recorder. A useful permanent record of the irradiation intensity was recorded by this system. Prior to colony irradiation the thermopile was mounted in a holder on the optical bench with all of the sensitive elements in the irradiation plane. Triplicate intensity measurements were taken before and after each irradiation experiment to insure that the intensity had not changed significantly. The intensity measurements were reproducible after the xenon lamp was prewarmed at least 1 hr before colony irradiation. Arc wandering was also minimized by the warm-up period but was not completely eliminated and required periodic minor adjustment at the entrance slit.

**Colony Irradiation.** Following the 10-day growth period in darkness, the colonies were marked by drawing a line on the bottom of the Petri dish along the growing hyphal front. This was necessary to align the photosensitive hyphal front properly with the square monochromator beam. The colony was then mounted in a large lens holder which was attached to an optical bench. This insured three things: the square monochromator beam was perpendicular to the colony, the colony was always the correct distance from the monochromator, and the same area of each colony would be irradiated. The growing hyphal front in the center of the colony approximately bisected the square beam from the monochromator. Exposure times were measured with a timer and manually controlled by means of a shutter at the exit slit. Following exposure to the required irradiation, the colony was incubated in darkness for an additional 7 days to allow for the development of perithecia along the irradiated hyphal front. The number of perithecia that developed along the exposed hyphal front were counted and formed the basis for construction of dose-response curves.

Two control groups were used which included colonies that were exposed to the red safelight only and those that received no irradiation. There were no detectable differences between the two control groups, indicating that the organism was not sensitive to prolonged exposure to the red safelight or possible background irradiation.

In all experiments where the exposure time was varied, the intensity was held constant at 143 ± 5 μw cm⁻². Since the Petri dish lids were not removed during colony irradiation, a correction for lid absorption was made prior to irradiation. To check reciprocity, neutral density filters were employed at the exit slit and a constant irradiation time of 200 sec was used at 440 nm. Reasonable agreement was found between dose-response curves derived from constant intensity and constant time experiments at 440 nm, although the maximum dose did not exceed 3 × 10⁶ ergs cm⁻².

**Perithecial Counting and Dose-Response Curves.** At least six colonies were individually irradiated for each exposure time. Exposure times varied with wavelength but were generally not over 5 min. Prior to counting, the colonies were flooded with 95% (v/v) ethanol to collapse the mycelium in the irradiated area. This also facilitated counting by placing the perithecia in one plane on the agar surface. The irradiated hyphal front was examined for perithecia at ×30 using an eyepiece grid (17.6 mm⁻¹) in a binocular dissecting microscope. The perithecial ridge that formed in response to irradiation was centered across the grid. The number of perithecia that developed in each of four grid areas was counted. The four grid values obtained for each colony were averaged to give the mean number of perithecia per 17.6 mm² per colony. The six colony values obtained for each exposure time were then averaged to give the mean number of perithecia per 17.6 mm² per exposure time. The six values obtained were used in constructing a dose-response curve for each wavelength. Each dose-response experiment was performed three times except for 440 nm which was performed five times.

Dose-response curves were determined at 10-nm intervals from 340 nm to 510 nm. Although the control colonies were kept in darkness, a few colonies perithecia formed in the photosensitive area. These perithecia were counted as described and subtracted from the mean values obtained for the exposed colonies. The resulting response values were plotted versus the exposure time in seconds for each wavelength. The dose-response curves assumed an approximately sigmoid shape.

**Action Spectrum Computations.** A standard perithecial response to irradiation was selected after examining the dose-response curves. The standard response was selected so that it would intercept the linear portion of all the dose-response curves. The standard response selected was 30 perithecia per 17.6 mm², which was about one-half the maximum response obtained for the most active wavelengths.

The standard response dose (intensity × time) for each wavelength was calculated and corrected using 440 nm as the wavelength of maximum effectiveness. The corrected standard response dose was then converted to quanta per cm² and the reciprocal plotted showing the relative quantum effectiveness versus wavelength of irradiation. Each point in the action spectrum curve presented in Figure 3 indicates the derived average relative quantum effectiveness from three independent experiments for each wavelength in addition to the standard error (s). Standard error was determined from s = s/√n where s is the standard deviation and n = 3 for all wavelengths except 440 nm where n = 5.

**RESULTS**

The photoresponses of the fungus are shown in Figure 1. Figure 1 shows (A) an unexposed 10-day-old colony with the hyphal front marked, (B) maximal perithecial formation from 5-min irradiation at 360 nm, (C) a low dose response of a double perithecial ridge, and (D) an unexposed 17-day-old colony. The double ridge shown in Figure 1C was characteristic of low doses only. The secondary ridge was not counted and disappeared at higher doses. Also, in Figure 1B, although a perithecial response occurred around the periphery of the square monochromator beam, only the perithecia developing on the exposed hyphal front could be quantitatively satisfactorily.

A comparison of the dose-response curves for four different wavelengths are presented in Figure 2. These determinations represent individual curves for each wavelength where the irradiation intensity was held constant at 143 ± 5 μw cm⁻². The dotted line indicates the standard perithecial response line from...
which each standard response dose was computed. The time required to achieve the standard response at 500 nm was over 10 min for this particular dose-response curve. The average of the five corrected standard response doses for 440 nm was $59.49 \times 10^3$ ergs cm$^{-2}$.

The action spectrum is presented in Figure 3. Each point represents the average relative quantum effectiveness derived from the independently conducted experiments. The standard error is shown as a line through each point. Longer exposures (over 20 min) to wavelengths from 510 to 560 nm, 600 nm, 650 nm, 700 nm, and 800 nm were unable to produce the standard perithecial response, and there was no evidence of any significant stimulation at these wavelengths.

The action spectrum indicates a broad peak of activity near 360 nm and a sharper peak near 440 nm. A smaller region of activity occurred near 480 nm. Regions of minimal activity occurred near 350 nm, from 390 to 410 nm and 470 nm. There are possibly shoulders of activity near 430 and 460 nm.

If the slopes of the linear portion of the dose-response curves are plotted versus wavelength, the resultant action spectrum curve does not differ greatly from the one shown in Figure 3. There were differences in the relative peak heights but the
Fig. 2. Examples of dose-response curves for four wavelengths obtained by varying the exposure time at a constant irradiation intensity of $143 \pm 5 \mu W \text{ cm}^{-2}$. Each point represents the average number of perithecia formed per 17.6 mm$^2$ in six replicates. The broken line indicates the standard response which was used to determine the standard response doses for action spectrum calculations.

Fig. 3. Action spectrum for photoinduced perithecial formation in *Nectria haematococca* var. *cucurbitae*. Each point represents the mean relative quantum effectiveness derived from three independent dose-response experiments except for 440 nm which is the mean from five experiments. The standard error is indicated for each point.

maxima and minima remained in the same positions. A similar situation occurred if the standard perithecial response for the action spectrum calculations was based on 20 or 40 perithecia.

**DISCUSSION**

Comparisons of the available action spectra data for fungal sporulation reveal essentially two different photo-absorbing systems. One is a far UV-absorbing system in the region from 230 to 350 nm (32). The other is a near UV-blue-absorbing system known for many different organisms. While the far UV action spectra data are important in partially explaining far UV sensitivity in several fungi and suggest a possible mode of action by the formation of compounds that stimulate sporulation, they do not account for reports of near-UV blue sensitivity (4-6, 8, 10, 12, 15, 19, 21) or red sensitivity (cf. 6) in other fungi.

The near UV-blue-absorbing system deserves special attention because it is known to mediate biological responses in various organisms (13). These responses include: the stimulation of carotene biosynthesis in *Fusarium aquaeductuum* (21), stimulation of oxygen uptake in *Chlorella pyrenoidosa* (20), potential of oxygen evolution in *Acetabularia crenulata* (29), plastid movement in *Lemma trisulca* (35), retardation of flower opening in *Oenothera* (22), phototropic responses of *Avena* coleoptiles (31), polarotropic response of chloronema of the fern *Dryopteris filix-mas* (27), and polarotropism of Liverwort germ tubers (28), and phototropic bending of sporangiphores of *Phycomyces* (10) and *Pilobolus* (19). Thimann (30) noted that the action spectra of some responses are similar showing peaks near 445 nm, 472 nm, a shoulder at 425 nm, minima at 400 nm and 460 nm with a steep fall to nearly zero from 510 to 550 nm. The similarity of the action spectrum for photoinduced perithecial formation in *N. haematococca* var. *cucurbitae* to those mentioned above, strongly suggests a common photosensitive mechanism.

The near UV-blue asexual photoresponses in a few fungi have been examined. Action spectra for asexual reproduction (conidia) were determined for *Trichoderma* sp. (5, 12) in the near UV-blue region from 350 nm to 550 nm. Peaks of activity occurred near 380 and 440 nm with a minimum near 400 nm. Essentially no sporulation occurred from 525 to 1100 nm. Komagai and Oda (15) reported four distinct peaks at 320, 380, 430, and 480 nm. They also bring out the fact that blue light, effective in promoting sporulation in *T. viride*, appears to be inhibitory for asexual photosporulation in *Alternaria* (1) and *Alternaria solani* (17). Sporulation in *Stephylium botryosum* (16) and a circadian conidiation rhythm in *Neurospora crassa* (23) are also inhibited by near UV-blue irradiation. The best available data seem to present a curious situation in which near UV-blue irradiation either inhibits or stimulates asexual sporulation depending upon the fungus under investigation.

The nature of the photoreceptor molecule in the near UV-blue region is controversial (3, 30). The argument centers on a flavin or a cis-carotenoid as the active pigment, although a flavin hypothesis has been favored by some investigators (3, 19-21). In this connection, a heterothallic strain of *N. haematococca* var. *cucurbitae* was treated with a flavin inhibitor to gain evidence that a flavoprotein photoreceptor molecule may be involved in perithecial formation (33). Perithecial formation was inhibited by the compound atebrin and was reversed by direct application of riboflavin to the fertilized colonies. A riboflavin photoreceptor was concluded to be correlated with the photorequirement in the heterothallic strain. It should be noted, however, that atebrin has been found to be a relatively nonspecific enzyme inhibitor, binding with proteins and the reversal of atebrin inhibition by addition of flavins may be attributed to complex formation between the flavin and atebrin rather than reconstitution of the holoenzyme (14, 34). Also, other evidence indicated the mode of action of atebrin in *E. coli* may be by impairment of DNA replication (7). Similar objections may be made for the use of diphenylamine to reduce the carotenoid content in fungi since diphenylamine reacts with DNA (24). The absorption spectra of several flavoproteins have been reported (18), and there is some agreement with the action spectrum of *N. haematococca* var. *cucurbitae*. However, it is clear that the use of pigment inhibitors and action spectroscopy alone are not able to resolve unequivocally the problem of determining the photoreceptor in this photosensitive organism.

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**LITERATURE CITED**

ACTION SPECTRUM OF PERITHECIAL FORMATION