Induction and Quantification of Microsclerotia in *Rosellinia necatrix*

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ABSTRACT

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A new method was developed for the quantification of microsclerotia produced by *Rosellinia necatrix*. The method, which is based on extraction of melanin from microsclerotia, provided a good correlation between the numbers of microsclerotia and the amount of extractable melanin. Red, blue, and fluorescent (daylight-type) illumination induced microsclerotium formation, whereas near-UV light and darkness depressed the morphogenetic process. Scanning electron micrographs revealed that the microsclerotia were undifferentiated, irregular, and rough, being composed of interwoven hyphae.

Additional key words: action spectrum, morphogenesis, *Dematophora necatrix*.

The fungus *Rosellinia necatrix* Prill. (imperfect stage *Dematophora necatrix* Hartig) causes severe damage to various plant and fruit trees, especially apple trees (10,12). It is characterized by a white mycelium, hyphae with pyriform swellings immediately above the septum, and the production of sclerotia which enable the fungus to survive in the soil between seasons (3). In culture, on potato dextrose agar (PDA), the fungus produces scattered black microsclerotia, about 98 × 130 μm in size, which tend to unite and form microsclerotial sheets (12,14). *R. necatrix* also produces synnemata on infected roots in moist chambers (14).

The research presented herewith was carried out to determine the influence of light and substrate on microsclerotium formation, and to study the ultrastructure of these resting bodies. Since it is difficult to count the numerous microsclerotia, a new method for quantitative analysis was developed.

MATERIALS AND METHODS

The fungus *R. necatrix*, isolated from infected roots of apple trees, was grown on potato dextrose agar (PDA). A mycelial disk, 4 mm in diameter, taken from the margin of a 7-day-old culture was transferred to petri plates each containing 15 ml of PDA with an initial pH of 5.8.

A new method for the quantification of microsclerotia by measuring their melanin content was developed. Cultures were boiled in water and the mycelium bearing microsclerotia, was lifted intact from the melted substrate. The mats were suspended in 20 ml of 1 N KOH, boiled for 2 hr and then centrifuged at 10,000 *g* for 15 min. The extracted melanin solution was titrated with 4 N HCl to obtain pH 6.0–6.5 and to precipitate the melanin. The precipitate was centrifuged at 10,000 *g* for 15 min, dried at 60 C for 24 hr, and weighed. It was then dissolved again in 4 N KOH and its absorption spectrum was tested by a UV-vis-Varian Spectrophotometer Model 635 (Varian Techtron Pty. Ltd., Melbourne, Australia).

The effect of light quality on formation of microsclerotia was determined by exposing colonies to red, blue, near-UV, and unfiltered fluorescent (daylight-type) light. The exposure to the different light wavebands was continuous during 30 days. Source of radiation for the red, blue, and unfiltered light was a white fluorescent lamp (Izrom 40 W white 4500° K, Izrom Electrical Industries Ltd., P.O. Box 1414, Jerusalem, Israel) with an intensity of 1800 lux, with or without filters. The filter used for the red was red-221, for the blue was dark blue-264, both were cellophane gelatin filters (Carrens Production, Bates Lighting Co., Los Angeles, CA 90029, USA) which transmit wave lengths ranging

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600–700 nm (peak, 650 nm) and 350–530 nm (peak, 440 nm), respectively. Source of the near-UV radiation was a black-light fluorescent lamp (Philips, TL 40 W/08RS, F 40 BLB, 6 K; Philips, Eindhoven, The Netherlands) which emitted radiation ranging 300–400 nm (peak, 350 nm). In order to obtain the same light intensity, 2.6×10^3 erg/cm^2/sec, measured by a Model 65 Radiometer (Yellow-Springs Instrument Company, OH 45387, USA), petri plates were placed at a distance of 35 cm from the light source in the artificial daylight and red illumination treatments, and 15 cm in the blue and near-UV ones. Plastic petri plates (AS/Nunc, Kamstrup, DK-4000 Roskilde, Denmark) covered with lids were used for the illumination treatments. The percentage of light transmitted through the plate lids at different wave lengths was: at 350 nm 70%; higher than 350 nm, 90–100%; at 310 nm, 80%; and lower than 310 nm, 0%, measured by the UV-vis-Varian Model 635 spectrophotometer. All experiments were carried out at 23 ± 1 C and repeated at least twice, each time in five replicates.

Microsclerotia were prepared for scanning electron microscopy in the following manner: mycelium and microsclerotia were placed in an ultrasonic disintegrator (MSE, London, England) for 2 min. The microsclerotia, separated from the mycelium by filtration and lyophilization for 2 hr, were then dried, put on specimen stubs, covered with gold film in an SEM coating unit (Model E5000; Polaron Equipment Ltd., Watford, England), and observed, at 25 KV, under a Cambridge-180 Stereoscan (Cambridge Scientific Instruments Ltd., Royston, England).

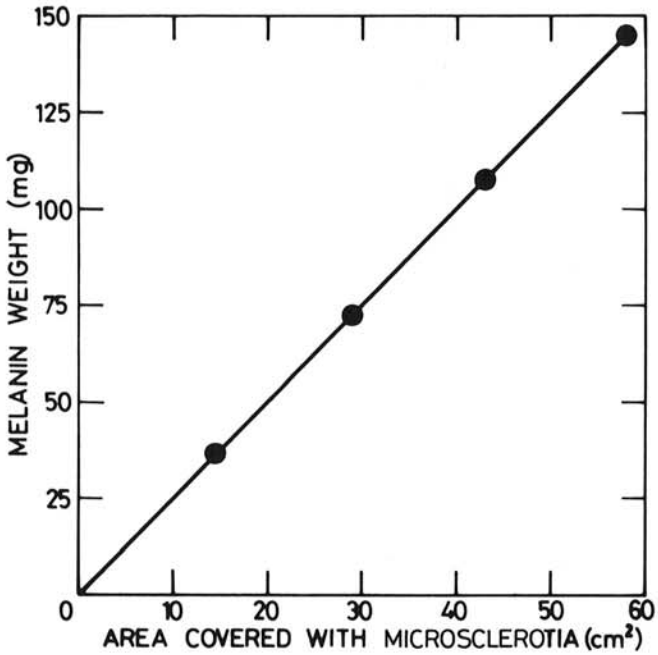

Fig. 1. The relationship between microsclerotium formation of *Rosellinia necatrix* and melanin production. On PDA, about 7,850 microsclerotia per square centimeter were present when melanin weight was determined 30 days after plate inoculation. Every data point number represents the average of five replicates.

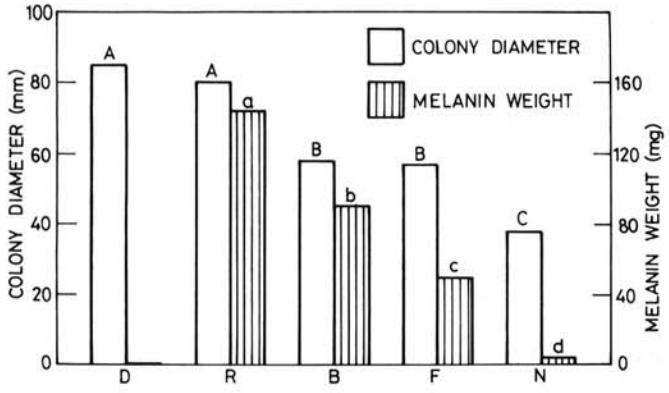

Fig. 2. The effect of light, at different wave lengths, on fungal growth and melanin production in *Rosellinia necatrix:* D=darkness, R=red light (600–700 nm), B=blue light (350–530 nm), F=fluorescent (daylight-type) illumination (380–700 nm), and N=near-UV (300–400 nm). Colony diameter and melanin weight were determined 8 and 30 days, respectively, after illumination treatments. Every number represents the average of five replicates. Different letters above the bars are assigned to means significantly different at P=0.05.

RESULTS

The fungus *R. necatrix* was grown on different synthetic media (Medium-A [1], Joham [8], Czapek [15], and Richard [6]) to find a defined medium inducing the formation of microsclerotia. None of the media tested induced the production of microsclerotia. Least fungus growth was observed on Czapek medium, while the Joham medium enhanced mycelial growth. Moreover, the addition to the

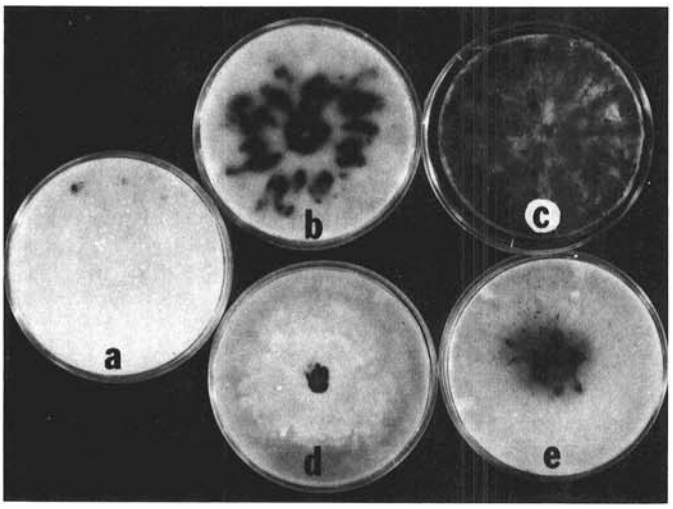

Fig. 3. The effect of light, at certain wave lengths, on fungal growth and the induction of microsclerotia: (a) darkness, (b) blue light (350–530 nm), (c) red light (600–700 nm), (d) near-UV irradiation (300–400 nm), and (e) fluorescent (daylight-type) illumination (380–700 nm).

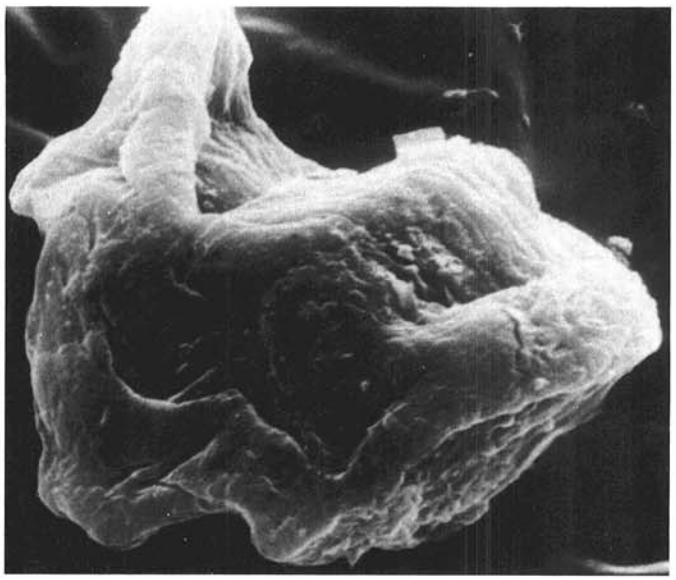

Fig. 4. A scanning electron micrograph of a *Rosellinia necatrix* microsclerotium showing its irregular shape and rough surface (× 1,000).

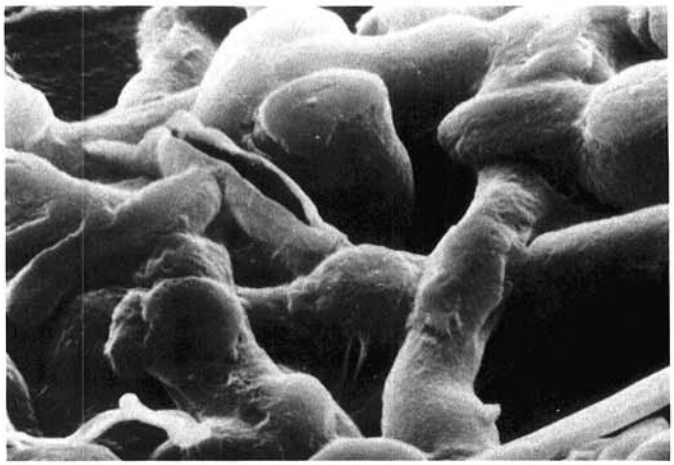

Fig. 5. A scanning electron micrograph showing the interwoven hyphae in a microsclerotium of *Rosellinia necatrix* (× 3,000).

Joham medium of various sources of carbohydrates (glucose, maltose, fructose, lactose, sucrose, sorbose, mannitol, cellulose, or pectin), nitrogen (KNO_3, $NaNO_3$, NH_4NO_3, or ammonium citrate), amino acids (alanine, arginine, asparagine, lysine, methionine, serine, threonine, or tyrosine), and vitamins (biotin, inositol, pyridoxine, thiamine, calcium pantothenate, or nicotinic acid) affected mycelial growth variously but had no effect on the formation of microsclerotia.

A good correlation was found between the numbers of microsclerotia in culture (Figs. 1 and 3) and the amount of extractable melanin (Figs. 1 and 2). Exposure of colonies grown on Joham or PDA medium to light depressed fungal growth except for red light (Fig. 2). Near-UV irradiation was most inhibitory. None of the light treatments affected microsclerotium formation in the Joham-medium-grown colonies, while, certain wave lengths—red, blue and unfiltered fluorescent light markedly increased the formation of microsclerotia in colonies grown on the PDA. Near-UV radiation and darkness depressed microsclerotium formation (Figs. 2 and 3).

Microsclerotia of *R. necatrix*, examined under a light and a scanning electron (SEM) microscope, were rough and irregular bodies (Fig. 4), composed of a compact mass of interwoven hyphae with no differentiated cells (Fig. 5).

DISCUSSION

R. necatrix is a pathogenic fungus which, due to its microsclerotia, may survive in the soil for many years. In this study, microsclerotia were not formed in colonies grown on various synthetic media in darkness; however, when the fungus was grown on PDA and exposed to red (600–700 nm), blue (350–530 nm), or artificial daylight (380–700 nm) formation of microsclerotia was significantly increased. No microsclerotia were produced under near-UV irradiation or in the dark. Similarly, McClellan et al (13) and Kaiser (9) found that red light induced microsclerotia formation in *Verticillium albo-atrum* Reinke & Berth., though in this fungus, darkness was also found to induce the morphogenetic process. Leach (11) found that near-UV light depressed formation of microsclerotia. Brandt (2) noted that under illumination, *Verticillium* produced more microsclerotia when grown on an undefined medium, such as PDA, than it did when grown on a defined medium. These results are similar to those obtained by us, indicating that microsclerotia formation in *R. necatrix* was induced by the undefined medium (PDA), but not by any of the tested defined media.

Quantitative assessment of microsclerotia, produced in culture, was carried out by melanin extraction, since melanin was found only in the microsclerotia (not in the mycelium). Fig. 1 reveals a good correlation between the number of microsclerotia produced on PDA and the amount of extractable melanin. This can, therefore, serve as a general method for the quantification of resting structures, such as sclerotia, chlamydospores, and melanin-containing mycelium. Scanning electron microscopy shows that, in contrast with the sclerotia of *Sclerotium rolfsii* Sacc. (5), the microsclerotia of *R. necatrix* are irregular in shape and much less differentiated, being similar to the sclerotia produced by *Rhizoctonia solani* Kühn and *V. albo-atrum* (4,7). The role of microsclerotia in the survival of *R. necatrix* in the soil and their importance in pathogenicity still has to be studied.

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