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GRADUATE COLLEGE

PHYSIOLOGICAL, CHEMICAL, AND GENETIC STUDY OF PIGMENT PRODUCTION BY *ARTHRODERMA BENHAMIAE*

A DISSERTATION

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HAMID MAJID GHANI

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OF PIGMENT PRODUCTION BY

ARTHRODERMA BENHAMIAE

APPROVED BY

DISSERTATION COMMITTEE

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CHAPTER I

INTRODUCTION

Pigments produced by different fungi have been utilized by mycologists as an aid in their identification and characterization. Dermatophytic fungi are no exception and many produce pigments.

Earlier work on Dermatophyte pigmentation tends to fall into two categories: Physico-chemical analysis of pigments and factors affecting pigment production. Conn (13) stated that pigment production is fairly constant for any strain and that pigmentation may have more diagnostic value if the emphasis is laid on the chemical nature of the pigment produced rather than on the color manifested. Silva (48) mentioned that the expression of color varies not only with the individual isolate and with its genetic stability but also, to a large extent, with the culture medium on which it is grown. Since natural media contain complex substances, variations may occur between different

batches of media and results in variation in the quality and intensity of the colors displayed by a given isolate. Therefore, for pigment production to be useful in classification, or in genetic analysis, each organism should be examined repeatedly while growing on chemically defined media and under carefully controlled environmental conditions. In this way various nutrients could be evaluated for their influence on pigment production.

Numerous workers have cultured various dermatophytes on synthetic media and have investigated external factors which influence pigment production. Extensive studies in this field were done by Tate (53), Goddard (22), Mosher et al (38), Peck & Rosenfeld (41), Robbins (45), Lewis & Hopper (33), Robbins & Ma (42), Robbins & McVeigh (43), Burkholder and Moyer (9), Bocobo et al (8), McVeigh et al (35), Benham (3, 4), Stockdale (52), Swartz et al (51), Silva (48, 50), and Georg (20, 21). These studies clearly indicated that the type and color of pigments produced by dermatophytes depend upon the kind and amount of the nigrogen and carbon sources. Other factors involved are light, oxygen, humidity, temperature, pH and trace elements.

A study of the effect of each amino acid alone on pigment production seemed essential. Silva (48) studied pigment production by T. mentagrophytes using chemically defined media containing individual amino acids as the

only nitrogen sources. Her results showed that the pigment produced differed in color depending on the kind of amino acid used. Robbins & Ma (42) studying the growth factors for T. mentagrophytes observed yellow pigmentation with alanine, reddish-brown with glycine, valine, histidine and serine, and brown with arginine and asparagine. Five amino acids gave little or no improvement in growth. These were tryptophane, threonine, methionine, lysine and hydroxyproline. The latter was found to inhibit the growth of five dermatophytes investigated by Robbins et al (43). All of the sulfur-containing amino acids supported growth poorly or not at all.

Silva (48) studied the nutritional factors affecting pigment production by four different sp. of dermatophytes, including T. mentagrophytes. Experiments with single amino acids indicated that each species had differing amino acid requirements for pigment production.

In view of the considerable body of knowledge regarding the effect of nutritional factors on T. mentagrophytes, it would seem that this organism provides an excellent model in which to study the biosynthesis of pigments and their roles in fungal metabolism.

Many workers, including Wirth et al (56), Mier (37), McCabe & Mier (34), Baichwal & Walker (2), Zussman et al (59), Koehen et al (30), Ito et al (23, 24, 25), Fujii et al (19), and Nozawa (38) have demonstrated that the pigment of

dermatophytes is not a single substance, but a mixture of several different compounds as shown by various techniques of chromatography.

The significant differences in the chromatograms of the pigments of several species of dermatophytes examined justifies the use of such analytical techniques as an aid in dermatophyte classification in addition to morphological identifications (25).

There seems to be no agreement concerning the chemical nature of dermatophyte pigments. Several investigators, including Tate (53), Thompson (54), McCabe and Mier (34), Rowe et al (46), Koehne et al (30), Mier (37), Ito et al (23), Fujii et al (19), Nozawa (39), and Just et al (29) have suggested that the pigments of dermatophytes are closely related chemically and are quinoids, possibly anthraquinone or naphthoquinone derivatives.

Evidence supporting this point of view is based on results obtained from color changes of the pigments with alkali, their pH indicator properties reversible reduction with sodium hydrosulfite and oxidation by atmospheric oxygen, and ultraviolet and infra-red spectra. Other direct evidence in support of this view is the isolation of a naphthoquinone pigment from T. megninii, called xanthomegnin by Blank et al (7). Just et al (29) determined the chemical structure of xanthomegnin by using nuclear magnetic resonance spectrum and other methods. The structure was

found to be

(-) 3,'3-bis/2-methoxy-5-hydroxy-7(2-hydroxypropyl) -8-carboxy-1,4-naphthoquinone lactone7.

xanthomegnin was later isolated from other species of dermatophytes including T. rubrum; T. violoceum; and M. cookei (24, 19, 56).

A different point of view as to the chemical nature of dermatophyte pigments is held by Zussman et al (59) and Merz et al (36) who claimed that dermatophyte pigments are melanoid in nature. Evidence for this point of view is based on the presence of nitrogen in the pigment and finding a high specific activity following growth in the presence of randomly labeled tyrosine or tyrosine labeled in the 2-position with C^{14}. Another line of evidence came from the isolation and characterization of a diffusible pigment produced by a strain of T. mentagrophytes which was claimed to be dopachrome (2-carpoxy 2,3-dihydroindole-5, 6-quinone), an intermediate in melanin biosynthesis (36).

The biological functions of dermatophyte pigments are not clearly known at present, but evidence from other groups of fungi and bacteria suggests that some of these pigments may be respiratory. Ito et al (26) made an attempt to investigate the intracellular distribution of the pigments produced by the dermatophytes. They found that approximately 80% of the total amounts of the pigment was localized in the mitochondrial fraction. They suggested,

based on evidence from bacteria and plants (14, 15), that these pigments play some active role in respiration, namely in the electron transfer chain. However, to evaluate the role of pigments in metabolism more accurate information must be obtained on their chemical structure and properties and on the requirements and mechanism for their production.

In 1967 Ajello et al (1) discovered the perfect state of T. mentagrophytes which was named Arthroderma benhamiae. Since this discovery, very little genetic work has been done with this organism, although it has most of the advantages which led fungal geneticists to work extensively with Neurospora and Aspergillus. Ajello (1) did a genetic study on the inheritance of the compatibility factors in A. benhamiae and found that compatibility is controlled by a single locus with 2 alleles (A,a) or (+,-). Similar work was done by Kown (31) on Nannizzia and the results also showed that mating competence is controlled by a one locus-2 allele system.

One major factor which contributed to the geneticists lack of interest in dermatophytes in the past was the phenomenon of pleomorphism. However, this problem was overcome when Castellani (10, 11) discovered a simple method to maintain fungal stocks as a thoroughly washed conidial suspension in glass distilled water, thus minimizing pleomorphism and maintaining a constant genotype for each isolate for indefinite periods of time.

Ploemorphism is not the only mutation found in dermatophytes, but it is the most frequent. Weitzman (57) had shown that peomorphism was the result of one or more gene mutations in loci which control conidial production and which were inherited in a simple Mendelian fashion. Many investigators found that pleomorphism was accompanied by loss of pigmentation and an increase in the ability to utilize an inorganic nitrogen source. This may indicate that the genes which control pigment and conidial production were linked to each other. It also indicates the relationship between these two traits and nitrogen utilization (6). Weitzman (57) and El-Ani (16, 17) suggested that the term pleomorphism should be discarded in favor of the term mutation. Bistis (5) suggested that mutation and positive selection were responsible for the high frequency of pleomorphism in T. mentagrophytes.

Evidence furnished by Goddard (22), Robbins et al (42), Nickerson (40), McVeigh et al (35), and Bistis (6) showed that pleomorphs were more efficient in utilizing an inorganic nitrogen source and had a greater growth potential than the granular forms. Therefore, it was suggested (6) that pleomorphism not be considered synonymous with degeneration, but rather that it be considered the result of mutation and positive selection. Spontaneous mutation plus selection could account for the appearance of pleomorphic patches in every colony while a given mutant appears rarely. Pleomorphism was different from the phenomenon of sectoring,

which was due to heterokaryosis (i.e. some nuclei mutate and each mutated unit was segregated into a hyphal system which grows and develop into a sector colony).

The aim of this study was to furnish information on the specific nutritional precursors required for the biosynthesis of pigments and to attempt to define their chemical structure and their role in metabolism. The increased interest in physiological and chemical studies of this sort provides additional criteria for the classification of dermatophytes.

Another purpose was to determine whether there were simple physiological and genetic differences that exist between our 3 parental isolates and to utilize them to understand the way in which pigments were synthesized and inherited. Insofar as I am aware, no comparable report for any species of dermatophytes has been previously recorded.

CHAPTER II

MATERIALS AND METHODS

Cultures used:

Dr. Ajello kindly furnished us with the compatible strains of A. benhamiae mentioned in the discovery of the sexual state of this organism (1). Three strains were used: TM-9 which was isolated originally in California from a horse, and was found to carry the (A) or (+) compatibility allele. TM-17 was isolated originally in Illinois from a dog and was found to carry the (a) or (-) compatibility allele. TM-20 was isolated originally in Missouri from a human source and was found to carry the (A) or (+) compatibility allele. On modified Sabouraud's dextrose agar the zoophilic strains were found to produce powdery to granular colonies with brownish-red to red-wine pigment in the subsurface mycelium.

Single Spore Isolates:

Single spore isolates of the above 3 strains were obtained by growing each strain in small prescription bottles containing modified Sabouraud's dextrose agar medium supplemented with 1% yeast extract. A spore suspension was made by adding a sterile solution of saline and 0.025% Tween 80 to the bottles and agitating with a Vortex mixer to release conidia. The spore suspension was then

centrifuged at 5,000 rpm for 20 min. and the supernatant was decanted. The spores were resuspended in the sterile solution of saline and Tween 80 and the suspension was filtered 3 times through a layer of sterile absorbent cotton (18) to remove mycelia and to obtain a mixture of microconidia and macroconidia. To remove the macroconidia, the spore suspension was allowed to settle for 15-20 min. The saline-Tween 80 mixture facilitated their sedimentation (31). A drop from the surface of the suspension was transferred with a sterile Pasteur pipette to the micromanipulator chamber and, by using the suction and the pumping devices of the micromanipulator, a single microaleurospore was picked up and transferred to a modified Sabouraud's dextrose agar plate. Germination began after 18 hours when the plate was incubated at 30°C.

Maintaining Stocks:

The single spore isolates of the above 3 strains of A. benhamiae and of the monoascospore isolates were maintained in stock using the Castellani method (10) to prevent pleomorphism. This was done by keeping a thoroughly washed microconidial suspension of each isolate in sterile cotton plugged test tubes containing 10 ml of glass distilled water.

Mating of P_1 and Production of Ascigerous State:

The ascigerous state was obtained by using sifted soil baited with horse hair. The soil was autoclaved on 3 successive days at 120°C for 30 minutes. The hair was autoclaved once. A thick spore suspension of each mating type was made and aliquot of every 2 compatible strains was poured onto the hair-baited soil. The plates were wrapped with aluminum foil and incubated in the dark at room temperature for 3 weeks. Cleistothecia were collected after 3 weeks and any adhering conidia were removed by gently rolling the cleistothecia across the surface of firm water agar with a fine needle. These cleaned cleistothecia were then transferred to another plate containing 4% water agar and kept there until needed for micromanipulation.

Micromanipulation Techniques:

A single cleaned cleistothecium was transferred to a small sterile shell vial containing 2-3 drops of sterile distilled water and crushed open by squeezing it against the wall of the vial. A drop of snail enzyme (Glusulase, Endo Lab) was added to soften the asci wall for 15-30 min (28). A set of large; 4 x 2 x 0.5 cm Mycosel agar blocks was prepared and each block was placed on a sterile Shoemaker slide. Another set of small; 1 x 2 x 0.5 cm Mycosel agar blocks was prepared and each block was placed in the

depression of the Shoemaker slide. With the aid of a sterile Pasteur pipette, a microdrop of the asci suspension was transferred and placed in the center of the small agar block and the asci were allowed to settle as the water evaporated. The slide was then set on the stage of a Leitz micromanipulator and, by using its suction and pumping devices, one mature ascus was transferred to the large agar block, close to the edge. The ascus was then allowed to settle as the water evaporates. Dissection of the ascus was performed under 100 X magnification by pressing on its surface with a microloop made of glass. The pressure applied on its surface was sufficient to burst it open and the ascospores were released. With the aid of the microloop, the ascospores were dragged on the surface of the large agar block a distance of 1 cm between individual spores. The Shoemaker slide was then removed and placed in a moist chamber consisting of a sterile glass petri dish and a wet filter paper. The ascospores germinated after 36-48 hours of incubation at 30°C. The germinated ascospores were cut out separately on small agar blocks and were transferred to modified Sabouraud's dextrose agar plates.

Determination of Ascospores Compatibility:

This study was carried out to test and classify the F_1 stock as to its mating type factor. Individual ascospores isolated from each ascus were backcrossed to parental TM-17 to determine the type of compatibility

system. Crosses which produced fertile cleistothecia were regarded as (A) or (+), while those which produced no fertile cleistothecia were regarded as (a) or (-).

Physiological Study on Parental Stock:

Such a study was necessary to determine how various amino acids would affect the development of pigments and to discover genetic and physiological variations between the parental isolates.

1. Preparation of the Inoculum:

   The inoculum of each strain was prepared as a microcomidial suspension in glass distilled water. This was done as mentioned previously in the procedures for maintaining stocks and spore isolation.

2. Preparation of the Basal Medium:

   Optimum conditions for growth and elaboration of pigments by T. mentagrophytes were obtained by using a previously developed, double-strength medium (48) consisting of 40 gm glucose, 0.1 gm $MgSO_4 \cdot 7H_2O$, 100 ml Sorensen's phosphate buffer, and 900 ml glass distilled water. The pH of the buffer was adjusted to 7.0. Amino acids were used as the nitrogen sources and were added singly in such amounts as to ensure a nitrogen concentration of 0.2 gm/liter of basal medium. The glucose was sterilized separately to prevent caramelization and thus preventing discoloration of

the medium and lowering its pH. Prior to autoclaving the initial pH of the medium was adjusted to the required level by using either 0.1 N HCl or 0.1 N NaOH. The presence of the buffer in the medium prevents any variability in the color of pigments due to changes in the pH of the medium during growth of the organisms.

3. Inoculation of Media:

Ten ml of each medium were dispensed in 150 x 20 mm tubes. The tubes were inoculated with 0.5 ml of the spore suspension and were incubated under constant shaking for 2 weeks in a New Brunswick shaker set at 250 rpm at 30°C. On these media the fungi grew in the form of pellets with a ring of mycelium on the wall of the tubes. Pigments were found not only on the pellets themselves but also occasionally in the medium.

4. Controls:

All cultures were run in triplicate and all the chemicals used were of C.P. grade. The glassware was cleaned with acid and thoroughly rinsed with tap and distilled water. As a positive control, pigment and growth on each amino acid was compared with pigment and growth on mineral basal medium alone and mineral basal medium plus 0.2% NH_4Cl. This was done to ensure that no nitrogen source or

other nutrients were transferred from the inoculum to the medium.

Physiological Study on Monoascospore Isolates:

This study was done to test and classify the F_1 stock as to color factor. Each monoascospore isolate was checked for the kind of pigment it produced as shown previously. l-leucine was used because the parental strains showed physiological and genetic differences on media containing this amino acid. Controls containing media inoculated with parental spore suspensions were used for comparison and the results were recorded. Other kinds of controls were the same as above.

Pigment Extraction:

The organism was killed by adding merthiolate (1/10,000) to the submerged cultures. The contents of the tubes were filtered after 48 hours and the filtrate was discarded. The mycelium was washed several times with distilled water, scraped from the surface of the filter, collected in extraction thimble, and placed in a Soxhlet extractor. Three hundred ml of petroleum ether (B.p. 60-80 c) was added to the extractor and refluxed for 6 hours to remove lipids from the mycelium. The petroleum ether was then decanted and replaced with 300 ml of acetone containing 1% of 1 N Hcl. A considerable amount of pigment was dissolved in the acetone after 6 hours refluxing in the extractor.

The extract was collected in clean test tubes and the acetone was removed by evaporation under vacuum using an Evapo-Mix apparatus (Buchler Instruments).

The residue was dissolved in ethylene dichloride, transferred to a separatory funnel, and then washed repeatedly with acidified water (1% of 1N Hcl) until the water became ninhydrin negative. The ethylene dichloride was removed by evaporation under vacuum and the residue was washed repeatedly with cyclohexane, followed with carbon tetrachloride to remove lipids.

The residue was dissolved in 1 ml chloroform, then 10 ml of methanol was added to precipitate pigment fractions which were insoluble in methanol. If a precipitate was formed, it was separated from the supernatant by centrifugation (5,000 rpm for 20 min), washed 3 times with methanol, and then redissolved in chloroform.

Pigment Purification

Separation of pigment components was done with thin-layer chromatopgraphy. Silica gel G plates were activated by heating at 110-120°C for 45 minutes. The tanks were equiliberated for 2 hours with benzene-methylethylketone-formic acid (75 : 24 : 1 V/V %).

Ascending chromatography was performed for 40 minutes. During this time the solvent front migrated about 15 cm. The plates were air-dried and examined under visible and Wood's U. V. lamp (long W. L.).

Pigment Characterization:

This study was carried out to determine some of the chemical properties of the purified pigments obtained from the brown and the yellow cultures of A. benhamiae.

Preliminary identification and differentiation of these pigments is facilitated by studying their pH indicator property, differences in solubility in organic solvents, photosensitivity and reactions with reagents specific for certain chemical compounds or certain radicals.

Not all the pigments extracted from the dermatophytes act as pH indicators. Moreover, pigments which act as pH indicators produce different colors when sprayed with strong acids and strong alkali such as 2N H_2SO_4 and 2N NaOH. This variation in color may indicate a difference in the chemical structure of these pigments.

Difference in solubility also may indicate a difference in the chemical nature of the pigments. All of the pigment fractions were tested for their solubility in chloroform, acetone, ethyelene dichloride, glacial acetic acid and methanol. Only one fraction was found to be insoluble in methanol.

The oxidation-reduction property of the pigments was tested by spraying the chromatograms with 50% sodium hydrosulfite solution. Reducible pigments change their color or become colorless when treated with this reducing agent but will regain their original color slowly when

exposed to atmospheric oxygen (37). A test specific for polyhydroxy anthraquinone pigments was done by spraying the chromatograms with 0.5% magnesium acetate in methanol and drying in an oven at 90°C for 5 minutes. A positive test was indicated when the color of the pigment is changed (47). Photosensitive pigments lose their color when exposed to light but retain their color when kept in dark containers. Only one fraction was found to be photosensitive (Table 9). Ito (27) reported that the production of melanoid pigments by fungi was inhibited or delayed when they were grown in media containing sufficient concentrations of ascorbic acid which probably acts as a reducing agent. Ascorbic acid was added to our chemically defined medium in a concentration of 0.2 mg/100 ml of the medium. The results of its effect on pigment production by A. benhamiae were recorded in Table 9.

CHAPTER III

RESULTS

In Table 1 the results show that strain TM-20 never produced a brown pigment when grown on the chemically defined medium regardless of the kind of nitrogen source used. The only color produced by this strain was yellow.

Strains TM-9 and TM-17 did produce a brown pigment in addition to the yellow when certain amino acids were used as the nitrogen source. The results also show the inhibition of growth of all 3 strains by sulfur-containing amino acids. Other amino acids such as histidine, glycine, lysine and serine also supported poor growth and pigment production. It is clear from the results that the nitrogen sources used affected the kind of pigment produced by each strain.

The data in Tables 2 and 3 show that our mating results were in agreement with the work of Ajello et al (1) who found that the compatibility was inherited through a 1-locus -2- allele system. The tables show a 1:1 segregation of the (A) and the (a) allele.

Failure of TM-20 to produce the brown pigment may be due to a single or multiple gene mutation. A cross was

made between this strain and TM-17 and the monoascospore isolates were tested for their ability to produce pigments on the amino acid L-leucine.

The results as shown in Table 4 indicated a 1:1 segregation of the brown and the yellow pigments. However, unless we map the mutations or obtain more information about the history of these strains, we can not determine whether this change in the phenotype was due to a single or multiple gene mutations.

Table 5 is a combination of the data obtained from Tables 3 and 4. It shows the classes of phenotypes obtained in a cross heterozygous for 2 loci (AY x aB).

Table 6 shows the classes of tetrad produced in a cross heterozygous for 2 loci. Three classes of tetrads are possible: Parental)PD), recombinant (NPD), and tetratype (T).

It is important here to consider each tetrad as a unit, either PD or NPD. The meiotic products of each tetrad should not be scored separately. The reason is that the formation of each tetrad arises as a result of an independent event, crossing over or no crossing over. On the other hand, the 4 meiotic products of a tetrad are not formed independently of each other.

The ratio NPD/PD=1 is an indication of independent assortment. When there is linkage between 2 loci, the frequency of NPD recombinant tetrads falls below the frequency of the PD tetrads. This is because PD frequency

represents non-recombinants, while NPD frequency represents tetrads in which double crossing over have occurred.

The tetratype (T) frequencies can occur for both linked and independently assorting genes. In independent assortment, tetratype (T) arise through second division segregation between one of the genes and its centromere. If the two non-linked genes are close to their centromere the frequency of (T) will be low. If the genes are far from the centromere, the frequency of (T) increase. When there is linkage, the tetratype (T) frequency represents tetrads in which single crossing over have occurred. In this case, even if the distance between the 2 genes is great and the frequency of double cross over is very high, we can still be fairly sure that the frequency of NPD will never rise above 1/4 the frequency of (T).

The results in Table 6 showed a good evidence for linkage between the compatibility gene and pigmentation gene. The unequal ratio of PD/NPD tetrads was indicative of linkage. A good evidence for independent assortment is found in the approximate equal frequencies of PD/NPD, and in an NPD/T ratio that is significantly greater than 1/4. The percentage of recombination can be calculated from the results of Table 6 as follows:

$$\frac{NPD + 1/2\ T}{PD + NPD + T} \times 100$$

$$= \frac{0 + 2}{10} \times 100$$

$$= 20\%$$

Table 7 shows the results of chromatographic analysis of pigments obtained from the yellow culture TM-20. One yellow spot (Fraction A) demonstrable with visible light was obtained.

Table 8 shows the results of the chromatagraphic analysis of the pigments obtained from brown cultures of TM-9 and TM-17. A yellow spot (Fraction A) demonstrable with visible light was obtained. The chemical characteristics of this fraction were similar to fraction (A) obtained from the yellow cultures TM-20 (Table 7). Both fractions had the same RF value (0.96) and both were not pH indicators. A red spot (Fraction B) visible with U.V. illumination was also obtained. However, the color changed quickly to yellow and later disappeared indicating sensitivity to light or to oxygen or both. This spot was believed to represent the yellow, methanol non-soluble, fraction because the result of the chromatographic analysis of this fraction was the same. Other pigments obtained include a red (Fraction C_1) and a blue (Fraction C_2) spots which were both visible under U.V. illumination.

The data in Table 9 show that fraction (A) which was isolated from both the brown and the yellow cultures differed chemically from fraction (B) which was isolated from the brown culture by methanal precipitation. Fraction (A) was not a pH indicator and it did not react with magnesium acetate, a reagent specific for the detection of anthraquinoid pigments which contain at least one hydroxyl group in the alpha position (47). Fraction (A) was inhibited by adding L-ascorbic acid in concentration of 2 mg/100 ml to the basal medium used in our physiological study. This indicated that this fraction is probably melanoid in nature (27).

TABLE 1. EFFECT OF AMINO ACIDS ON PIGMENT PRODUCTION BY *T. mentagrophytes* STRAINS TM-9, TM-17 and TM-20

Amino Acids	TM-9	TM-17	TM-20
L-leucine	B	B	Y
L-alanine	Y	B	Y
L-arginine (free base)	B	B	W
L-arginine-HCl	W	W	W
L-glutamic acid	B	Y	Y
L-glutamine	Y	Y	Y
L-aspartic acid	B	B	Y
L-asparagine	B	Y	Y
L-isoleucine	Y	Y	W
Glycine	Y	W	W
L-lysine	Y	Y	Y
DL-serine	Y	W	W
DL-threonine	-	-	-
L-methionine	-	-	-
L-histidine	-	-	-

B=brown Y=yellow W=colorless -=no growth

TABLE 2. DETERMINATION OF COMPATIBILITY FACTORS OF MONOASCOSPORE ISOLATES IN TM-9 x TM-17 MATING

Ascus	spore #1	spore #2	spore #3	spore #4	spore #5	spore #6	spore #7	spore #8
1	A	A	a	a	A	A	A	-
2	a	a	A	A	A	A	-	-
3	a	a	A	A	a	A	-	-
4	A	A	A	a	a	a	-	-
5	a	A	A	a	A	a	-	-
6	A	A	a	A	a	a	a	A
7	A	a	A	a	a	a	a	A
8	a	A	a	A	A	A	a	a

TABLE 3. DETERMINATION OF COMPATIBILITY FACTORS OF MONOASCOSPORE ISOLATES IN TM-20 x TM-17 MATING

Ascus #	spore #1	spore #2	spore #3	spore #4	spore #5	spore #6	spore #7	spore #8
1	A	A	a	a	a	A	A	-
2	A	a	A	a	A	a	A	-
3	A	A	A	a	a	a	-	-
4	a	A	a	a	A	A	-	-
5	a	A	a	A	A	a	A	a
6	A	a	A	a	a	A	A	-
7	A	A	a	a	A	a	-	-
8	A	a	A	A	a	A	a	a
9	A	a	a	A	a	a	-	-
10	A	a	A	A	a	a	-	-

TABLE 4. TYPES OF PIGMENTS PRODUCED BY TM-20 x TM-17 MONOASCOSPORE ISOLATES USING L-LEUCINE AS A NITROGEN SOURCE

Ascus #	spore #1	spore #2	spore #3	spore #4	spore #5	spore #6	spore #7	spore #8
1	Y	B	Y	B	B	Y	B	-
2	B	B	B	Y	Y	B	Y	-
3	Y	Y	Y	B	B	B	-	-
4	B	Y	B	B	Y	Y	-	-
5	B	Y	B	Y	Y	B	Y	B
6	B	B	Y	B	Y	Y	B	-
7	Y	Y	B	B	Y	B	-	-
8	Y	B	Y	Y	B	Y	B	B
9	Y	B	B	B	Y	Y	-	-
10	Y	B	Y	Y	B	B	-	-

TABLE 5. TETRAD ANALYSIS OF MONOASCOSPORE ISOLATES OBTAINED FROM THE CROSS TM-20 x TM-17

Ascus #	spore #1	spore #2	spore #3	spore #4	spore #5	spore #6	spore #7	spore #8	tetrad class
1	AY	AB	aY	aB	aB	AY	AB	-	T
2	AB	aB	AB	aY	AY	aB	AY	-	T
3	AY	AY	AY	aB	aB	aB	-	-	PD
4	aB	AY	aB	aB	AY	AY	-	-	PD
5	aB	AY	aB	AY	AY	aB	AY	aB	PD
6	AB	aB	AY	aB	aY	AY	AB	-	T
7	AY	AY	aB	aB	AY	aB	-	-	PD
8	AY	aB	AY	AY	aB	AY	aB	aB	PD
9	AY	aB	aB	AB	aY	aY	-	-	T
10	AY	aB	AY	AY	aB	aB	-	-	PD

TABLE 6. TETRAD CLASSES OBTAINED FROM THE CROSS TM-20 x TM-17

TETRAD CLASS			
PD	NPD	T	
AY	AB	AY	
AY	AB	aY	
aB	aY	AB	
aB	aY	aB	
6	0	4	Total = 10

TABLE 7. CHROMATOGRAPHIC ANALYSIS OF PIGMENTS FROM YELLOW CULTURES OF T. mentagrophytes

Fraction	color under U.V. illumination	color under visible illumination	RF
A	Blue	Yellow	0.96

TABLE 8. CHROMATOGRAPHIC ANALYSIS OF PIGMENTS FROM BROWN CULTURES OF *T. mentagrophytes*

Fraction	color under U.V. illumination	color under visible illumination	RF
A	Blue	Yellow	0.96
B	Red-Yellow	-	0.85
C_1	Red	-	0.30
C_2	Blue	-	0.10

TABLE 9. CHARACTERIZATION OF PIGMENT FRACTIONS (A) AND (B) OBTAINED FROM BROWN AND YELLOW CULTURES OF *T. mentagrophytes*

Test	Fraction A	Fraction B
pH indicator property	-	+
Solubility in methanol	+	-
Light sensitivity	-	+
Sodium hydrosulfite reagent	+	+
Methanolic magnesium acetate	-	+
Ascorbic acid inhibition	+	-

CHAPTER IV

DISCUSSION

When morphological criteria are used in the classification of dermatophytes problems are encountered regardless of whether we use characteristics of the perfect or the imperfect state. The characteristics of the imperfect state are not an accurate index of phylogenic relationships because several perfect species possess identical conidial forms. Furthermore, some perfect species have more than one conidial form.

An important perfect state characteristic for differentiation among the Ascomycetes is the nature of the ascocarp. Unfortunately, ascocarp morphology has little value in the separation of species in the genus Arthroderma because there is uniformity in the structure of the peridium as well as the asci and ascospores. The dimension of the asci and ascospores in all members of this genus are 5 x 6 u and 2 x 2.5 u respectively.

Morphological characteristics of the asexual spores are of value in the differentiation of some genera and species of the dermatophytes. However, many species of Trichophyton are not known to produce macroconidia, and

the microconidia of certain species are so similar as to be of no value in classification. Moreover, microconidia may be absent in pleomorphic colonies and the cultures will resemble each other so closely that they can not be identified. Morphological characteristics of the same isolate may also vary with age and culture media.

When isolates present a highly varied morphology and reveal no distinctive spore form or spore arrangement, identification must depend on physiological characteristics. Physiological tests also serve to confirm identification based on morphological criteria and permit classification in the absence of distinguishing structures. They may also lead to the discovery of media which favor the production of asexual and sexual structures.

Complete dependence on physiological characteristics in dermatophytes taxonomy will lead also to chaos because, as in the case with morphological criteria, variation within and between species is great. Pleomorphic forms are more efficient than the granular forms in utilizing inorganic nitrogen sources and are more resistant to the inhibitory action of sulfur-containing amino acids and hydroxyproline (43).

Many workers have emphasized the importance of pigmentation in dermatophyte taxonomy. Pigments were regarded in the past as a single chemical substance and changes in their shade were thought to depend solely on

the pH and the oxidation-reduction potential of the culture. No chemical or genetic evidence was presented to support this idea.

In the early fifties, chromatographic analysis of pigments began and the data obtained revealed that the dermatophytes and other fungi produce a complex mixture of pigments. Some of them acted as pH indicators while others did not. Pigments which acted as pH indicators were found to be different from each other because they produced different colors when treated with strong acids and alkali or when reduced with sodium hydrosulfite. These variations in color ruled out the idea that pigments constitute one chemical entity.

There are two conflicting ideas regarding the chemical nature of pigments. Some workers claim that dermatophyte pigments are anthraquinoid or naphthoquinoid in nature while others claim that they are melanoid in nature. However, the evidence furnished by each group was obtained by analyzing only certain fractions of the pigment and neglecting other fractions. No exclusive work was done to determine the chemical nature of every fraction produced by each species studied or to determine strain variations within a single species of the dermatophytes.

Ito et al (23) advocated the idea of using the chemical analysis of pigments as a tool to classify the dermatophytes down to species level. However, our results

indicated that this character was of limited value even when we were dealing with variants of the same species. Moreover, pleomorphic forms were reported to lose their ability to produce pigments as well as other morphological characteristics.

TM-20 never produced a brown culture when grown on chemically defined media regardless of the nitrogen source used. Only yellow cultures were obtained. Strains TM-9 and TM-17 produced brown cultures in addition to yellow cultures. The production of the brown cultures depended on the kind of nitrogen source used. It was clear from these results that these three strains differed in their ability to produce pigments even when grown under the same environmental conditions. The difference might be due to genetic factors and not to environmental factors.

The chemical analysis of the pigment produced by TM-20 showed only the presence of one yellow fraction (fraction A). Strains TM-9 and TM-17 were also found to produce fraction (A) plus three other fractions (B, C_1, C_2). Fraction (B) was separated as an amorphous solid by precipitation with methanol. This fraction was believed to be anthraquinoid in nature because it was a pH indicator, had an oxidation-reduction property and gave a positive test when treated with magnesium acetate.

The data obtained from the genetic analysis showed that at least 2 different genes were involved in pigment

production. Strain TM-20 was believed to differ from strain TM-17 by at least one gene mutation. However, unless we know the genetic history of these strains we can not tell exactly how many genes were responsible for this change in their phenotype. The data of the genetic analysis showed also that the genes for pigment production were linked with the compatibility gene and the distance between them was estimated to be 20 map units.

CHAPTER V

SUMMARY AND CONCLUSION

Taxonomic confusion has resulted from variations in the morphological and physiological characteristics of the dermatophytes. Because of this inconsistent behavior, no single property is considered to be reliable for the classification of the genus Trichophyton down to species level. The most reliable identification probably should be based on a combination of all the morphological and the physiological characteristics. Therefore, the statement that chemical analysis of pigments alone can be used as a tool for the differentiation of dermatophytes is unwarranted.

Chemical and genetic evidence was introduced to show that strain variation did exist in A. benhamiae regarding the nature of chemical substances produced by pigmented strains. These substances include a yellow pigment visible under visible light and three fluorescent fractions visible under U.V. illumination. Genetic analysis indicated that at least two different genes were involved in production of these substances. Each gene may produce one fraction or a group of chemically related pigment

fractions. The genes for production of these substances were shown to be linked with the compatibility gene and the distance between them was estimated to be 20 map units.

The chemical analysis of the substances produced by TM-20 and TM-17 furnished an additional evidence for strain variations. The isolation of a common visible yellow pigment (fraction A) suggested the presence of a common gene between these two strains. The absence of the other fractions from TM-20 alone indicated the probability of mutations in one or more genes.

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