CULTURAL CONSIDERATIONS IN ENDOTHIA PARASITICA STATE OF THE ART

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ABSTRACT.-- Factors *affecting in vitro growth, reproduction, and pigment formation by Endothia parasitica are discussed from a historical perspective. There also is a brief discussion on the methods currently used for sexual reproduction studies, vegetative compatibility, and conversion testing. The potential for the need of a selective medium for use in dissemination studies also is mentioned.*

The purpose of this "State of the Art" paper is to renew an awareness of factors that need to be considered when studying the cultural behavior of *Endothia parasitica.* This paper is not a review of all the literature on *in vitro* growth, reproduction, pigment formation, etc., of this fungus. Rather it is a discussion, from a historical point of view where possible, of some of the cultural and environmental factors that have been shown to affect *E. parasitica* and how this relates to present-day research. Until recently, all research was conducted with what are now considered normal-virulent isolates of this fungus; hypovirulent isolates are "abnormal" culturally. Therefore, the results cited from work done by the early workers are confounded by hypovirulence.

Even though the topics discussed in this paper are interrelated, they are arbitrarily divided. Part of the discussion will be centered on the affect of nutritional and environmental factors on cultural behavior, especially the formation of pigment and pycnidia. There also will be a brief discussion of the kind of pigments produced by this' fungus and the current in *vitro* methods used for sexual reproduction studies and vegetative compatibility and conversion testing. There also will be a brief mention for the need of a selective medium for use in dissemination studies, especially as related to hypovirulent isolates.

Cultural Characterics

General medium effects

Endothia parasitica grows readily *in vitro* and on a variety of substrates. This lack of fastidious growth requirements has advantages and disadvantages.

It is an advantage because the fungus grows so readily on such a variety of substrates, the choice of substrate is left to the researcher. Anderson and Rankin (1914) noted in the saprophytic condition this fungus seemed omnivorous. This lack of fastidiousness is a disadvantage, however, because little definitive work on the basic physiology of this organism has been conducted.

Early workers noted *E. parasitica* grew and sporulated on a large variety of substrates: sterilized root and twigs of a variety of tree and shrub species, bean plugs, carrots, potatoes, sweet potatoes, bread, corn meal, oat meal, rice, sugar solutions, bouillon, lima bean agar, oat agar, potato agar, corn meal agar, chestnut bark media, prune agar, starch, and Raulin's fluid to list a few (Anderson and Babcock 1913; Anderson and Rankin 1914; Clinton 1909; 1912a; 1912b; Cook and Wilson 1914; Heald et al. 1915; Shear and Stevens 1913; Sher et al. 1917). Yet with the tremendous diversity of substrates suitable for supporting growth of this fungus, relatively few have been used extensively and the cultural behavior of this fungus has been described in detail on still fewer. Much of the early cultural work was conducted in an effort to develop reliable cultural criteria that could be used to distinguish *E. parasitica* from several closely related *Endothia* species (Anderson and Anderson 1913); Clinton 1912a; Shear and Stevens 1913; Shear et al. 1917). These cultural parameters were especially valuable when the sexual stage of the species in question was not available (Shear et al. 1917),

A few of the culture media used by these early workers and a description of the cultural characters observed are going to be mentioned. Although the media they used may no longer be important, the types of cultural characteristics and their variability as affected by the test medium as well as the detailed observations of the cultural morphologies made are relevant to current work comparing normal and hypovirulent *E. parasitica* isolates.

Murrell commented (quoted in Anderson and Rankin 1914) that when *Diaporthe (Endothia) parasitica* was "grown in artificial culture, the mycelium is at first pure white, changing to a yellow with age and the fruiting pustules are a beautiful yellow." This description still is accurate for normal isolates of this fungus grown on some media. Anderson (1914) reported that "on potato agar the mycelium begins to turn yellow due to the production of pigment in the cells at the end of 4 to 6 days." "As the cultures age, the fungus often becomes purple or wine colored." Shear and Stevens (1913) reported when pycnospores of *E. parasitica* were streaked on a potato agar they described, (not the same medium used by Anderson 1913) "at the end of 3 or 4 days at room temperature, there was short, fluffy, white aerial growth along the streak. The surface of the mycelium was orange by transmitted light while by reflected light, it was between raw sienna and antique brown at the sides. Within 6 days the mycelium, especially at the base of the agar slant, took on a peculiar granular metallic appearance, due apparently, in part at least, to the character of the mycelium and in part to the minute water drops scattered over the surface. This portion of the culture was light orange-yellow by reflected light and orange by transmitted light. The peculiar surface appearance might perhaps be called "brassy". This metallic surface appearance has been found to be the most constant and reliable distinguishing character of *E. parasitica* on potato agar. In 12 to 14 days, small pycnidial pustules appeared in the upper portion of the tubes, and the agar just below the mycelium became warbler green." When grown on corn meal agar in Petri plates, these same authors (Shear and Stevens 1913) noted that "1-week-old cultures of *E. parasitica* covered about one-half the surface of

the medium. The outer margin was pure white, the remainder buff yellow, with a superficial white growth above and the medium uncolored. A few small pustules with spore masses occurred near the point of inoculation. Cultures 1 month old showed a compact growth nearly smooth in the surface. The superficial mycelium was pale yellow. The pale yellow-ocher spore masses were minute, very numerous and nearly covered the surface. The medium was slightly greenish about the sides of the flask just beneath the mycelium." The number and size of the pycnidia produced on this medium was considered to be an important character for distinguishing *E. parasitica* from other *Endothia* species (Shear and Stevens 1913). Clinton (1912a) also noted a yellow pigment developed on a lima bean medium but as the culture aged, the pustules became light chestnut brown. Later Shear et al. (1917) reported on additional cultural studies with species of *Endothia.* They noted the cultural characteristics produced by *E. parasitica* on corn meal agar in unslanted tubes were very reliable for distinguishing it from other *Endothia* species. They reported that after 6 to 8 weeks, there was "a scantly white growth of superficial mycelium, with several prominent pycnidial pustules clustered near the center area of a slightly darker shade than the raw sienna." In this same paper, they (Shear et al. 1917) also reported on the cultural characteristics of *E. parasitica* on a semi-defined, liquid medium, referred to modified Cooks medium No. II. After 1 month of incubation in 30 ml of this media in 100 ml Erlenmeyer flasks, the mycelial growth was "very abundant, closely matted, chiefly submerged but slightly aborescent in one or two small areas which remained above the surface." The color of the mycelium was a dark greenish brown (Shear et al. 1917).

Although descriptions of cultural characters on various substrates could continue; these examples demonstrate that the substrate affects cultural morphology and pigment formation in this fungus. Rather than being concerned about characterizing species of *Endothia,* today we are more concerned with the cultural variability that exists between and among normal and hypovirulent isolates of *E. parasitica* and how the chosen substrate affects this variability. Most workers in North America currently use Difco potato dextrose agar for these types of comparisons. Workers in Europe often use other media including Maltea-Moser agar. These media work well for distinguishing normal from hypovirulent isolates, but it is not always possible to make direct comparisons between European and North American work. In addition, if a potato dextrose agar medium other than Difco is used, cultural characters often are drastically altered. Even though there are culture media that work very satisfactorily for distinguishing between normal and hypovirulent isolates and among hypovirulent types, defined media should be developed that discriminate among these isolate types. This would improve repeatibility of results among workers and might provide information on the physiological differences that exist among these isolate types.

Pigment and pycnidia formation

In addition to the general affects the medium constituents have on the cultural behavior of *E. parasitica* and how this behavior is confounded by hypovirulence, factors affecting *in vitro* pigment and pycnidia formation warrant further consideration. The production of pigment and asexual reproduction are closely associated in this fungus. The characteristic yellow-to-orange color produced by normal isolates of this fungus on many media is thought to be similar to that responsible for the characteristic color of the stromata

in the bark (Anderson 1914). Even if the medium or the environmental conditions do not support intensive in *vitro* pigment formation, if pycnidia are produced, they usually are pigmented yellow-to-orange (Shear et al. 1917). Since the lack of pigment and normal pycnidia formation on Difco potato dextrose agar or Maltea-Moser agar is one *in vitro* criterion used to distinguish some hypovirulent isolate types from normal ones, the cultural, physiological, environmental, and biochemical factors affecting pigment formation and reproduction in this fungus need further study. In addition, when one considers there are hypovirulent isolates (the JR and North American types) that produce abundant pigment on Difco potato dextrose agar but do not produce normal pycnidia, the significance of understanding the mechanism of pigment formation and its association with the development of pycnidia is further reinforced.

Pigment formation. There was considerable interest in pigment formation by species of *Endothia* on various media by early workers. Some of this work regarding the affect of the medium on pigment formation already has been mentioned, so additional comments will be restricted to the types of pigments produced by *E. parasitica.* Pantanelli (in Anderson 1914 and Roane and Stipes 1978) noted the superficial mycelium in culture was bright yellow because of lipochrome drops in the cells. Later Anderson (1914) suggested the pigment was aurine rather than a lipochrome. He observed that as the cultures of *E. parasitica* aged on a potato agar, the pigment changed from a yellow to a purple. He related this change in color to change in the pH of the medium, acidic to alkaline. The production of pigment on a corn meal medium was one cultural criterion that could be used to separate *E. parasitica* from other closely related *Endothia* species (Shear et al. 1917). Hawkins and Stevens (1917) described pigments produced by three species of *Endothia* and concluded they probably were not aurine or lipochrome. Sands (1919) examined one of these pigments in detail. He assigned it the emperical formula $C_7H_5O_4$, concluded it was probably related to members of the pyrocatechin group, and named it endothine red. It was not until Shibata et al. (1953; 1955a; 1955b) began working with the pigments of *Endothia* species, however, that details of their chemistry were unraveled. Four pigments, skyrin, skyrinol, oxyskyrin, and rugulosin were identified in species of *Endothia* (Shibata et al. 1953; 1955a; 1955b). Skyrin, oxyskyrin, and sykrinol are 1,1-bisanthraquinones whereas rugulosin is a modified bisanthraquinone (Shibata 1967; 1973). Shibata (1967) developed a biogenetic-scheme for the production of these pigments from acetyl and malonyl coA. Production of secondary metabolites by the condensation of an acetyl unit with malonyl units is characteristic of fungi and often results in formation of pigments (Turner 1976). The metabolism of acetate by *E. parasitica* has been shown to shift towards the synthesis of bisanthraquinones and fatty acids during sporulation (McDowell and DeHertogh 1968).

Roane and Stipes (1978) reported skyrin, oxyskyrin and rugulosin were present in cultures of *E. parasitica* grown on a white corn meal medium. The one hypovirulent isolate they tested, EP-43, also contained these pigments. With the increased number of hypovirulent isolates that have been found, the constantly improving biochemical techniques, and the variation in pigment formation in culture among these isolates, additional work on the types of pigments present and their variation, if present, between and among normal and hypovirulent isolates of this fungus warrants further work. The results of such studies might provide additional criteria for distinguishing between

and among normal and hypovirulent isolate types and information on the metabolic and biochemical disturbances caused by hypovirulence.

Pycnidia formation-light effects. Light also has been shown to affect pycnidia and pigment formation in this fungus. Anderson (1914) stated "when plate cultures are grown in total darkness on chestnut agar, no pycnidia are developed, while on plates made at the same time and grown in the light, the usual rings of pycnidia appear." When cultures were incubated in darkness until the medium was about half-covered with mycelium and then exposed to light, "circles of pycnidia were developed, beginning with the ring which marked the outermost limit of the colony when removed from the dark chamber. The concentric rings which always appear on agar cultures are due to the alternation of night and day" (Anderson 1914). Yet, Shear et al. (1917) noted "it is evident that pycnidia are produced abundantly in total darkness on chestnut-twig agar as well as on other favorable media. There is no perceptible difference in amount of spore production or in the arrangement of pycnidia between cultures kept in total darkness and those kept in light during the day if the temperature and evaporation remain the same in both."

Leonian (1924) also reported light did not affect pycnidia formation on a glucose-malt extract-peptone medium. In contrast, Barnett and Lilly (1952) demonstrated pycnidia formation on a glucose-casein hydrolysate agar medium was influenced by the wave length, intensity and length of exposure to light. Total darkness decreased the number of pycnidia produced, but those formed were larger than those in continuous light. White to blue (400 to 500 um) light were most stimulatory for pycnidia formation. A few large pcynidia were produced in red light, similar in number and size to those produced in darkness, and yellow and green light had an intermediate effect (Barnett and Lilly 1952). Campbell (1967), in the most critical recent study on nutritional factors affecting *in vitro* sporulation, used a carefully prescribed light treatment. After the plates were inoculated, they were wrapped in two layers of aluminum foil and incubated at 25 C for 5 days. The cultures were then exposed for 15 minutes to fluorescent lamps (130 fc, Champion cool-white with emission peaks in the blue region of the visible spectrum). After 3 days of additional incubation in darkness, sporulation was evaluated. Light also has been shown to enhance sporulation on a Difco potato dextrose agar medium (Anagnostakis 1978), and Grente and Sauret (1978a; 1978b) reported that pycnidia production by normal isolates on a Maltea-Moser agar began on the third day when cultures were grown in alternating light and dark. A few hours of light were enough to initiate pycnidia formation on this medium. In continuous darkness, pycnidia were thinly distributed and only appeared after 7 days' growth. Pycnidia were formed after 15 to 20 days by white, hypovirulent isolates if the culture was exposed to light more than 50 per cent of the time. Only a few pycnidia formed and there was no distribution pattern present (Grente and Sauret 1978a; 1978b).

These results demonstrate light stimulates in *vitro* pycnidia formation, at least on some media, and that normal and hypovirulent isolates respond differently. The effect of light on pycnidia (and pigment) formation needs additional work, and the wave length, intensity, duration, etc., of the light should be closely examined. There are means to examine the effects of light on fungi much more critically than what has been reported with *E. parasitica* (Trione and Leach 1969; Tan 1978).

Pycnidia formation-nutritional effects. Leonian (1924) also reported that *E. parasitica* could be induced to produce pycnidia by manipulating the nutritional conditions of the medium. He used a technique with several fungi, which consisted of growing the fungus in a medium (glucose-malt extract-peptone for *E. parasitica)* that was suitable for vigorous vegetative growth. The mycelium was then aseptically transferred to a weak medium. Often pycnidia formation was stimulated by these fungi when transferred to the weak medium, or starvation conditions. With *E. parasitica,* however, only pycnidial initials formed when the mycelium was starved, but if this mycelium was placed in a favorable medium again, optimal sporulation occurred. More recently, Campbell (1967) demonstrated the concentration of the carbon source and the nitrogen source as well as the method used to sterilize the medium affected pycnidia formation. The carbon to nitrogen ratio did not affect sporulation within the ranges he tested, but the absolute qualities and the type of carbon and nitrogen used, determined the intensity of sporulation. The degree of utilization of a carbon source, especially fructose, was affected by the method of medium sterilization. He found pycnidial formation was optimal in the medium containing fructose as the carbon source, if the entire medium was filter sterilized, or if fructose was sterilized separate from the other medium constituents, either by autoclaving or by membrane filtration. Certainly additional work on the nutritional basis for pigment and pycnidia formation is justified. The results of this work and that with the effect of light, etc., should provide for a more complete understanding of the factors affecting pigment and pycnidia formation and how hypovirulence affects their formation.

Sexual Reproduction

Techniques have been developed that support *in vitro* sexual reproduction by *E. parasitica* (Anagnostakis 1979; Willey 1980). It is now possible to perform controlled crosses so studies can be conducted on the inheritance of genetic traits in this fungus. In addition, test crosses can be conducted between normal and hypovirulent isolates. Although such crosses have been attempted unsuccessfully, additional studies on the nutritional and environmental (light, temperature, etc.) factors that affect in *vitro* perithecia formation can now be conducted.

Vegetative Compatibility and Conversion

Vegetative incompatibility among *E. parasitica* isolates may present a serious problem for application of hypovirulence. Transfer of hypovirulence factors between normal and hypovirulent isolates requires hyphal anastomosis and cytoplasmic exchange between the isolates. Vegetative incompatibility apparently prevents the exchange of cytoplasm and results in the failure of some hypovirulent strains to cure virulent cankers (Anagnostakis 1978). The vegetative compatibility (v-c) type of normal isolates can be determined using the technique described by Anagnostakis (1977; 1978). The medium (Difco potato dextrose agar), cultural conditions, age of the inoculum, placement of the inoculum on the test media, etc., as described by Anagnostakis must be closely followed for reliable results. If there was a defined media that would work as well or better, the problem of variation in natural media could be eliminated and results among workers would be more comparable. A defined media, if there was one that would work well for discriminating between normal and hypovirulent isolates, also might function for v-c testing.

Conversion also will occur when compatible, normal and hypovirulent isolates are paired on agar media (Grente and Sauret 1978a; Northup 1981; Anagnostakis 1982). Conversion does not occur as readily between incompatible isolates. Grente and Sauret (1978a) suggest conversion does not occur here because anastomoses between incompatible isolates results in degeneration of the cytoplasm at the site of the hyphae fusion, and this prevents complete cytoplasmic exchange. Northup (1981) developed a defined medium and a technique for microscopic examination of individual anastomosis between isolates. Hyphae to peg and peg to peg types of anastomoses (Buller 1933) were observed to occur between both vegetatively compatible and incompatible isolates. Collapsed cells, observed with both light and scanning electron microscopy, were abundant in the hyphae immediately adjacent to the site of hyphal anastomosis between incompatible isolates. Similar shrunken cells also were noted by Anagnostakis (1982). Whether the abnormal appearance of these cells is associated with cytoplasmic degeneration which may prevent transmission of hypovirulence between incompatible normal and hypovirulent isolates is not known. Microscopic examination of hyphal anastomosis in this fungus needs further attention. There are media and techniques available now that are suitable for use in these types of studies (Northup 1981; Anagnostakis 1982). The results of these types of tests should provide for more basic understandings of the nature and control of the transmission of hypovirulence in this fungus.

Selective Medium

One of the questions facing people working with hypovirulence as a biocontrol for chestnut blight is how are hypovirulent isolates naturally disseminated. These isolates often do not produce many pycnidia in host bark and there is no evidence they produce perithecia in nature. Natural dissemination has occurred in Italy (Turchetti 1978), however, and is occurring in Michigan. Natural dissemination of artificially introduced hypovirulent isolates also has been reported in West Virginia (Willey this proceedings). Workers have suggested birds, various insects, and mites may function as carriers for this fungus (Anderson and Babcock 1913) and might effectively carry hypovirulent isolates within and among trees (Anagnostakis this proceedings; Wendt et al. this proceedings). *Endothia parasitica* spores have been washed from insects that were observed to visit cankers. But, however unlikely, there may be an air-borne propagule that is important for natural dissemination of hypovirulent isolates. In order to determine if such a propagule exists, the air must be sampled in areas where hypovirulent isolates are spreading naturally, Italy, Michigan, and West Virginia. There are numerous methods for sampling the air, but because of the tremendous diversity and number of fungal spores that probably would be encountered, it might be difficult to microscopically examine for the fungus directly, and it might be difficult to culture the fungus from the sampling device. To facilitate air sampling studies, the use of a medium that is selective for *E. parasitica*, especially hypovirulent types, might be helpful. Anderson and Babcock (1913) and Heald et al. (1917) used petri plates with agar media to sample for airborne *E. parasitica* spores. The medium used by Heald et al. (1917) was selective. It inhibited growth of bacteria, retarded growth of fast growing fungi, and allowed for good growth and easy identification of the chestnut blight fungus. The results of this work clearly demonstrated ascospores were present in the air in blighted chestnut stands, and these workers concluded ascospores were responsible for long-distance dissemination of this

fungus (Anderson and Babcock 1913; Heald et al. 1917). There are no other reports of the use of selected media until the recent report by Russin et al. (this proceedings). Until there is conclusive data explaining the mechanism of natural dissemination of hypovirulence, researchers must not rule out the possibility of some form of aerial dissemination, and the use of a selective medium might be valuable for such studies.

There probably are other cultural considerations that should have been addressed in this paper. However, if the reader has become only slightly more aware of the lack of information and understanding of the cultural behavior of *E. parasitica,* then the author's purpose will have been fulfilled. With the discovery of hypovirulence in this fungus and the unexplained cultural and pathogenic abnormalities caused by the hypovirulence factors, the lack of basic information is confounded. Challenges still remain therefore, for those interested in studying the cultural behavior of *E. parasitica* and how hypovirulence affects this behavior.

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