MYCELIUM PRODUCTION IN LIQUID MEDIA BY SEVERAL NORMAL, HYPOVIRULENT AND CONVERTED ENDOTHIA PARASITICA ISOLATES

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ABSTRACT.--Tests were conducted with several liquid media to determine if mycelium production could be used to differentiate between normal, hypovirulent, and in vitro converted isolates of Endothia parasitica. Difco potato dextrose broth generally supported more mycelium formation than any other medium tested. Arginine supported the production of more mycelium than any other amino acid tested and more than the inorganic nitrogen source, ammonium tartrate. Cellobiose, glucose and fructose supported more mycelium formation in a defined medium with arginine as the nitrogen source than other carbon sources tested. Usually those hypovirulent isolates that grow faster and are white or slightly pigmented on Difco potato dextrose agar produced more mycelium than those hypovirulent isolates that grow slower and pigment more intensely. Mycelium production varied among normal isolates in each medium, and that produced by converted isolates often differed markedly from that produced by either of the original isolates. Mycelium production by four isolates with identical genetic backgrounds but with varying dsRNA components varied in some defined media. The normal isolates often produced more mycelium than the hypovirulent isolates, but mycelium production did not differentiate among the hypovirulent ones. Although mycelium production varied among the test media and among isolates, this cultural characteristic could not be used to reliably differentiate between the normal and hypovirulent isolates tested. Mycelium production on certain media may have been used for quantitatively differentiating those hypovirulent isolates that grow more rapidly and produce slight pigment from those that grow more slowly and pigment more intensely.

Endothia parasitica, the fungus causing chestnut blight, nearly destroyed American chestnut as a forest tree in North America and caused severe losses to chestnut in Europe. Since the introduction of the fungus into North America in about 1904, many unsuccessful controls for the blight on American chestnut were tried, and interest in this problem waned. Recently however, there has been renewed interest in this disease. In Italy living blighted European chestnut trees were found with superficial healing cankers. Originally this was attributed to the development of resistance to *E. parasitica* in the host (Turchetti 1978). However, Grente and Berthelay-Sauret (1978a) recovered isolates of the chestnut blight fungus from these healing cankers that were less pathogenic than normal (N) ones, and possessed abnormal

cultural morphologies. When grown on a Maltea-Mosear agar, these hypovirulent (H) isolates often grew somewhat faster, sporulated less, and produced less pigment than N isolates (Grente and Berthelay-Sauret 1978b). By single sporing these isolates, an N and the slightly pigmented, white H isolate type were retrieved, as well as a slow growing pigmented type of H isolate, called JR (Grente and Berthelay-Sauret 1978b).

Hypovirulent isolates of *E. parasitica* also have been found in the United States (Day et al. 1977; Elliston 1978). These H isolates were obtained from American chestnuts that had survived the onslaught of chestnut blight and had cankers that were healing. The North American H isolates possess cultural morphologies on potato dextrose agar that differ from that of the N isolate types. Commonly they are pigmented but grow slower and sporulate less than N isolates.

To date, all H isolates tested contain dsRNA (Day et al. 1977; Elliston 1978) and its presence is thought to be responsible for the hypovirulence characteristics and the abnormal cultural morphologies. The nature and the mechanism of action of the dsRNA in H isolates, however, has not been fully characterized.

Workers have demonstrated (Anagnostakis 1978; MacDonald and Double 1978; Double personal communication) N and H isolates of *E. parasitica* can be readily differentiated when cultured on Difco potato dextrose agar (PDA). However these distinguishing criteria are subjective and dependent upon the cultural conditions used. Obviously the physiology of N and H isolate types must differ to account for the different cultural morphologies on PDA, but these differences cannot be adequately examined using media containing natural products. To date no studies on the specific physiological differences that exist between N and H isolate types using defined media have been reported. There are several reports, however, discussing the physiology of N isolates of this fungus.

Early reports indicated *E. parasitica* was capable of utilizing ammonium nitrogen and was partially deficient for thiamine and biotin (Lilly and Barnett 1951). Treggi (1954), using a Czapeks-Doxs medium, reported *E. parasitica* could utilize several amino acids as sources of nitrogen and if the concentration of glucose in this medium was increased, growth increased. Bazzigher (1958) reported that for this fungus to grow in a Knop-glucose solution, growth substances from chestnut bark were required. When the Knop-glucose medium contained bark extracts and thiamine, glutamic acid, aspartic acid, glutamine or asparagine were utilized effectively as nitrogen sources (Bazzigher 1958). He also showed biotin was required for growth on the inorganic nitrogen source, NH4NO3. Campbell (1967) reported the concentration of the carbon source (glucose or fructose) and the nitrogen source (asparagine) as well as the method of medium sterilization affected asexual sporulation by *E. parasitica.* Other work demonstrated a medium containing inorganic salts, glucose and thiamine supported good growth and asexual reproduction by this fungus, but when yeast and malt extract were added, growth and sporulation were enhanced (Puhalla and Anagnostakis 1971). Most recently, Uchida (1977) reported several *E. parasitica* isolates grew well in Richard's solution with glutamic acid, tyrosine, asparagine or methionine as nitrogen sources, but peptone supported optimum growth. He also reported the fungus grew well on a variety of mono-, di-, and

polysaccharides, but soluble starch was the best carbon source tested. With the resurgence of interest in the American chestnut blight problem and a general lack of information on the physiological differences that exist between N and H isolates, studies were initiated to determine: whether mycelium production in liquid media (dry weights) could be used to differentiate between N and H isolates of *E. parasitica;* whether medium constituents would differentially affect mycelium production among these isolates.

Materials and Methods

Sixteen isolates of *E. parasitica* were used in these tests: 16-15-1, an N isolate from West Virginia (grows somewhat slower on PDA than other N isolates); 6-3-1, an N isolate from West Virginia; 523, an N isolate derived from a single spore culture of H isolate 518 (Elliston 1981; 1982; personal communication); EP-49N, a single spore isolate of H isolate EP-49 with N culture morphology on PDA (Willey 1980); EP-43, a dsRNA containing H isolate (Day et al. 1977) that is fast growing and white or very slightly pigmented on PDA; EP-49, a dsRNA containing H isolate from Italy (Day et al. 1977) that is fast growing and slightly pigmented in PDA; EP-50, a dsRNA containing H isolate (Day et al. 1977) with cultural morphology quite similar to EP-49; 27-10, a dsRNA containing H isolate (Willey 1980) from Italy with cultural morphology on PDA similar to EP-49 and EP-50; EP-88, a dsRNA containing H isolate (Elliston 1978) that is slower growing and produces more pigment on PDA than EP-49, EP-50, or 27-10; EP-4, a dsRNA containing H isolate (Elliston 1978) that produces abundant pigment and grows slowly on PDA; 518, an H isolate from Michigan (Elliston 1978) that produces pigment, grows slowly on PDA and contains dsRNA components identified as Hm_1 and Hm_2 (Elliston 1981; 1982; personal communication); 524, an H isolate derived from a single spore of 518 that produces less pigment, grows faster on PDA than its parent isolate 518 and contains the dsRNA component identified as Hm_2 (Elliston 1981; 1982; personal communication); 544, an H isolate derived from a single spore of 518 that produces abundant pigment, grows somewhat faster on PDA than its parent isolate 518 and contains the dsRNA component identified as Hm_1 (Elliston 1981; 1982; personal communication); 16-15-1 plus EP-4, an *in vitro* convertant of N isolate 16-15-1 by H isolate EP-4; 16-15-1 plus EP-50, an *in vitro* convertant of N isolate 16-15-1 by H isolate EP-50; 6-3-1 plus EP-43, and *in vitro* convertant of N isolate 6-3-1 by H isolate EP-43.

All media tested, except Difco Bacto potato dextrose broth (PDB), contained a basic complement of constituents: 1.0 g KH_2PO_4, 0.5 g $MgSO_4$, 0.2 mg Fe^{++} ($FeSO_4 \cdot 7H_2O$), 0.2 mg Zn^{++} ($ZnSO_4 \cdot 7H_2O$), 0.1 mg Mn^{++} ($MnSO_4 \cdot H_2O$), 0.1 mg thiamine, and 1000 ml distilled water. The carbon and nitrogen sources tested, which varied according to the test, are presented in the results section. Except for media containing 10 g glucose and 2 g casein hydrolysate, 2 g peptone or 2 g yeast extract, all contained the amount of nitrogen provided by 2 g asparagine and the amount of carbon provided by 10 g glucose. The pH of all test media except those containing natural products was adjusted to 6.0 ± 0.1 with KOH or H_2SO_4. After pH adjustment, all media were dispensed into 250 ml Erlenmeyer flasks (25 ml/flask) and autoclaved. Agar plug inocula were placed in the flasks, one plug per flask with five replicate flasks of each medium per isolate. The resulting cultures were incubated at 25 C for 10 days in diffuse 12/12 light and dark. The resulting mycelium was harvested through a fine cloth, dried at 80 C and weighed to the nearest mg.

Analyses of variance were conducted and F-values calculated on mycelium production in each medium to determine if significant differences in mycelium production existed among isolates. In addition, Duncan's multiple range tests at 5 percent were conducted on mycelium production among media to determine which medium supported most mycelium production, and among isolates to determine if they could be separated into N or H isolate types using mycelium production (Steele and Torrie 1960; Snedecor and Cochran 1967).

Results and Discussion

Potato dextrose broth supported more overall mycelium production than all other media tested (Tables 1, 2, 3 and 4). Of the other media containing natural products (Table 1), the medium containing yeast extract as the nitrogen source supported less mycelium production than PDB, but more than the media containing casein hydrolysate or peptone as nitrogen sources (Table 1). The optimal mycelium production in PDB is not surprising. Difco PDA, a medium similar to PDB but containing Difco Bacto agar, also supports good growth of *E. parasitica* and is the medium of choice for growth of this fungus by many workers. In fact, the cultural characteristics produced on PDA serve as one *in vitro* standard for differentiating between N and H isolate types. The relatively poor performance of peptone in our test is at variance with those reported by Uchida (1977). Peptone generally is considered to be a good nitrogen source for fungi including *E. parasitica* (Shear et al. 1917), but in our tests, supported no more mycelium production than some of the amino acids. The differences in cultural conditions we used, compared to those of Uchida (1977) and Shear et al. (1917) might account for the difference in the results with peptone.

Table 1. Mean values for mycelium production (mg) among 16 isolates of *Endothia parasitica* grown for 10 days at 25 C in 25 ml of liquid media containing natural products

Medium	Dry weights[y/]
Potato Dextrose Broth	144a
Yeast Extract	121b
Casein Hydrolysate	89c
Peptone	75c

[y/] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Table 2. Mean values for mycelium production (mg) by 16 isolates of *Endothia parasitica* grown for 10 days at 25 C in 25 ml of a defined liquid medium containing glucose as the carbon source and one of six nitrogen sources

Nitrogen source	Dry weights[y/]
Arginine	82a
Asparagine	60b
Proline	52bc
Valine	41cd
Methionine	32d
NH_4 Tartrate	32d

[y/] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Table 3. Mean values for mycelium production (mg) by 16 isolates of *Endothia Parasitica* grown for 10 days at 25 C in 25 ml of a defined liquid medium containing fructose as the carbon source and one of five nitrogen sources

Nitrogen source	Dry weights[y/]
Arginine	76a
Proline	44b
Valine	41b
Methionine	29c
NH_4 Tartrate	21d

[y/] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Even though the mean value for mycelium production in PDB was 144 mg, the amount of mycelium produced by the individual isolates ranged from a high of 281 mg for isolate EP-49 to a low of 58 mg for isolate EP-4 (Table 5). With the exception of EP-43, those H isolates (EP-49, EP-50 and 27-10) that grow more rapidly and produce limited pigment on PDA produced more mycelium than those H isolates (EP-4, EP-518, EP-524 and EP-544) that grow more slowly on PDA and pigment more intensely. These results are not surprising when the relative amounts of radial growth on PDA by these isolates are compared. Mycelium production also varied significantly among N isolates, and 16-15-1, which grows somewhat slower on PDA than some other N isolates, produced less mycelium than two of the other N isolates tested, 6-3-1 and EP-49N. Converted isolate 16-15-1 plus EP-4 produced more mycelium than its original isolates and more than convertant 16-15-1 plus EP-50. Mycelium production did not discriminate between the four isolates (EP-518, EP-523, EP-524 and EP-544) with identical genetic backgrounds but with varying dsRNA components.

Potato dextrose agar is routinely used to discriminate between the isolates of *E. parasitica* containing these components, but the criteria Elliston (1981 this proceedings) uses to distinguish between isolates containing these dsRNA components, including radial growth on PDA, do not seem to be associated with mycelium formation in PDB (Table 5).

Table 4. Mean values for mycelium production (mg) by 16 isolates of *Endothia parasitica* grown for 10 days at 25 C in 25 ml of a defined liquid medium containing arginine as the nitrogen source and one of five carbon sources

Carbon source	Dry weights[y/]
Cellobiose	86a
Glucose	82a
Fructose	76ab
Pectin	64b
Soluble starch	37c

[y/] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Table 5. Mycelium production (mg) by 16 isolates of *Endothia parasitica* grown for 10 days at 25 C in 25 ml of potato dextrose broth

Isolates[y/]	Dry weights[z/]	Isolates[y/]	Dry weights[z/]
H EP-49	281a	C 6-3-1 + EP-43	135cde
C 16-15-1 + EP-4	253a	H 518	97def
H EP-50	237ab	H 524	97def
C 16-15-1 + EP-50	192bc	N 16-15-1	84ef
H EP-88	186bc	H 544	83ef
H 27-10	170c	N 523	78ef
N 6-3-1	155cd	H EP-43	63f
N EP-49N	139cd	H EP-4	58f

[y/] H, dsRNA containing hypovirulent isolates; C, converted isolate; N, normal virulent isolate.

[z/] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Arginine supported more mycelium production in the media containing glucose or fructose as carbon sources than the other amino acids tested and more than the inorganic nitrogen source, ammonium tartrate (Tables 2 and 3). Methionine and ammonium tartrate supported the least mycelium production

whereas intermediate amounts of mycelium were produced in the glucose-asparagine, -proline, and -valine media and in the fructose-proline and -valine media (Tables 2 and 3). These results are at variance with those of other workers. Asparagine (Bazzigher 1958; Uchida 1977) and methionine (Uchida 1977) were reported to support optimum growth by this fungus.

Cellobiose and glucose supported more mycelium formation in the medium containing arginine as the nitrogen source than pectin or soluble starch (Table 4). Fructose supported mycelium production in this medium equal to that produced in the cellobiose, glucose and pectin media (Table 4). Uchida (1977) reported *E. parasitica* grew in media with a variety of mono-, di- and polysaccharides as carbon sources, but soluble starch supported optimum growth. This carbon source supported the least mycelium production of the carbon sources we tested (Table 4). Pectin and cellobiose have not been tested as carbon sources by other workers, but since these materials are components of plant cell walls, they probably serve as carbon sources for this fungus growing in chestnut bark. With the inability of some H isolates (EP-43 and EP-4) (Double personal communication) to develop cankers in American chestnut, it was thought the inability of these isolates to establish a food relationship with the host might be associated with the lack of canker development. Our data do not eliminate this as a basis for the lack of canker formation, but since these isolates can use cellobiose and pectin as carbon sources the lack of a carbon source probably is not involved with their inability to form cankers.

Mycelium production in the glucose-, fructose- and cellobiose-arginine media also varied among isolates (Tables 6, 7 and 9). Those H isolates that grow more rapidly and are white or only slightly pigmented on PDA (EP-43, EP-49, EP-50 and 27-10), produced more mycelium in these media than those H isolates but grow more slowly and pigment more intensely (EP-4, EP-518, EP-523 and EP-524). This is the same type of difference noted in PDB (Table 5). Mycelium production among N isolates on these media also varied (Tables 6 and 7), similar to that observed in PDB. However, the amount of mycelium produced by the individual N isolates in the test media was not consistent. Normal isolates 16-15-1 produced less mycelium than 6-3-1 or EP-49N in PDB, but equal to or more than those isolates in the glucose- and fructose-arginine media. Mycelium production by converted isolates in the glucose-, fructose-, and cellobiose-arginine media often differed from each other and from that of the original isolates. The mycelium production of the four isolates with identical genetic backgrounds with varying dsRNA components differed on these media. The N isolate, EP-523, consistently produced more mycelium than the H isolates, EP-518, EP-524, and EP-544, but mycelium production did not discriminate among the H isolates.

Although less mycelium was produced in the glucose-valine medium than in any of the other media presented in this report, many of the same general mycelium production patterns present in the other media, were evident (Tables 5, 6, 7, 8 and 9). The H isolates that grow more rapidly and are white or slightly pigmented on PDA (EP-43, EP-49, EP-50 and 27-10) produced more mycelium than those H isolates (EP-4, EP-518, EP-524 and EP-544) that grow more slowly and pigment more intensely. There was less variation in mycelium production among N isclates in this medium than in the others, and mycelium production by N isolate EP-523 only differed from that produced by H isolates EP-518 and EP-524. Mycelium formation by the converted isolates often differed from one another and from one or both of the original isolates.

Table 6. Mycelium production (mg) by 16 isolates of *Endothia parasitica* grown for 10 days at 25 C in 25 ml of a defined liquid medium containing glucose as carbon source and arginine as the nitrogen source

Isolates[y/]	Dry weights[z/]	Isolates[y/]	Dry weights[z/]
H 27-10	182a	C 16-15-1 + EP-4	70fg
H EP-49	139b	N EP-49N	67fgh
H EP-50	127bc	C 16-15-1 + EP-50	62gh
H EP-43	122bcd	H EP-88	60gh
C 6-3-1 + EP-43	104cde	N 6-3-1	50gh
N 523	95def	H 524	39hi
N 16-15-1	77efg	H 544	21ij
H EP-4	72fg	H 518	11j

[y/] H, dsRNA containing hypovirulent isolates; C, converted isolate; N, normal virulent isolate.

[z/] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Table 7. Mycelium production (mg) by 16 isolates of *Endothia parasitica* grown for 10 days at 25 C in 25 ml of a defined liquid medium containing fructose as the carbon source and arginine as the nitrogen source

Isolates[y/]	Dry weights[z/]	Isolates[y/]	Dry weights[z/]
H EP-50	148a	N 6-3-1	58g
H 27-10	139ab	H EP-88	57gh
H EP-49	126bc	N 16-15-1	54gh
H EP-43	112cd	H EP-4	48gh
C 16-15-1 + EP-4	102de	C 6-3-1 + EP-43	43h
N EP-49N	89ef	H 524	25i
N 523	87ef	H 544	22i
C 16-15-1 + EP-50	85f	H 518	18i

[y/] H, dsRNA containing hypovirulent isolates; C, converted isolate; N, normal virulent isolate.

[z/] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Although these nutritional studies were not exhaustive, only 20 media were tested, and only a small number of *E. parasitica* isolates used, it is obvious the nutritional constituents of liquid media affected mycelium production (Tables 1, 2, 3 and 4). Potato dextrose broth generally supported more mycelium production than all other media tested, and glucose was equal to or better than the other carbon sources tested in the defined media. The amino

converting isolates, EP-4 plus EP-50, produced very different amounts of mycelium in PDB, but when they were used to convert this isolate, 16-15-1, the resulting convertants produced mycelium quite different from one another and from that of either of the original isolates (Table 5). Similar differences in mycelium formation by these convertants were evident in other test media (Tables 6, 7, 8 and 9).

Table 9. Mycelium production (mg) by 16 isolates of *Endothia parasitica* grown for 10 days at 25 C in 25 ml of a defined liquid medium containing cellobiose as the carbon source and asparagine as the nitrogen source

Isolates[y]	Dry weights[z]	Isolates[y]	Dry weights[z]
H 27-10	125a	C 16-15-1 + EP-50	93cd
H EP-49	122ab	N 16-15-1	92cd
H EP-43	117abc	C 6-3-1 + EP-43	89de
N 523	116abc	H EP-88	89de
N EP-49N	102abc	H 524	64e
N 6-3-1	97bcd	H 518	30f
H EP-50	97bcd	H 544	24f
C 16-15-1 + EP-4	96bcd	H EP-4	19f

[y] H, dsRNA containing hypovirulent isolates; C, converted isolate; N, normal virulent isolate.

[z] Different letters in column indicate means significantly different at the 5 percent level using the Duncan's multiple range test.

Elliston (1981 this proceedings; personal communication) suggests H isolates EP-518, EP-524, and EP-544 only differ in their dsRNA components, and the presence of these specific dsRNA's confers distinct cultural and pathogenic abnormalities to these isolates. It was unfortunate mycelium production would not discriminate between these isolates. It was hoped mycelium formation might serve as a tool for quantitative differentiation.

With the almost total lack of information on physiological differences that exist between N and H isolate types as well as within various H types, the results of this study can only serve as a starting point for additional tests. Such physiological studies should continue because results should provide a more complete understanding of the basis for the cultural and pathological abnormalities conferred on *E. parasitica* by the hypovirulence factor(s).

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