Genetics and Nutritional Requirements of Endothia parasitica

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ABSTRACT

A medium containing inorganic salts, glucose, and thiamine supports good growth and conidial production of *Endothia parasitica,* but the conidia require the addition of biotin or aspartate for germination. The pH optimum for mycelial growth is 4, and the fungus does not grow above a pH of 5.5. The optimal temperature range is 25-30 C. Light does not affect mycelial growth rate or pigment development, but is necessary for formation of conidia. Biochemical and morphological mutants isolated after irradiation with ultraviolet light were used to synthesize heterokaryons and putative diploids. Perithecia containing normal ascospores were formed in the laboratory on chestnut stem segments inoculated with genetically marked strains. Ascospore analysis suggests that *E. parasitica* is homothallic but will preferentially outcross. Phytopathology 61:169-173.

Additional key words: Heterokaryosis, parasexuality.

Endothia parasitica is the fungus which annihilated the American chestnut early in this century, and a definitive study of the infection was done by Anderson (1). The fungus can be grown on a variety of laboratory media, and will produce pycnidia in culture. Unlike pycnidia formed on the host, they are not in a stroma. The conidia are uninucleate, do not germinate in water, but do germinate on most laboratory media. They are long-lived and extremely resistant to drying. Perithecia are never formed on these media, forming on the host among the pycnidia within the fungal stromata below the host's bark. The ascospores are two-celled structures, each cell containing one to four nuclei. They are long-lived, and germinate readily even in water.

Bazzigher (2) noted that biotin, in combination with an inorganic nitrogen source, and thiamine were essential for germination of conidia. Aspartic acid or glutamic acid could substitute for the biotin and inorganic nitrogen. More recently, Campbell (3) examined the relationship between nutrition and conidial formation, but his studies did not extend to the factors which affect growth of hyphae.

Live chestnut trees in the field can be artificially inoculated with *E. parasitica,* but no inoculations with genetically marked stocks have been reported; consequently there is little or no information about the nuclear cycle of this fungus. In this paper we report further work on the nutrition of *E. parasitica* and a genetic study using ascospores formed in the field and in the laboratory. Mutants were used to demonstrate heterokaryosis and to study parasexuality in the fungus.

MATERIALS AND METHODS.—All strains used in this study were derived from infections of chestnut trees in the vicinity of New Haven, Conn. Strain EPB was retrieved as a large piece of mycelium under the bark of an infected tree. Strain 3.6 is a single conidial isolate from an ascospore of a natural infection. All biochemical and morphological mutants arose from 3.6.

Minimal medium (MM) was prepared from salt solution, 62.5 ml; thiamine, 2 mg; glucose, 10 g; and distilled water to 1 liter. Complete medium (CM) contained in addition Difco yeast extract, 2.5 g; and Difco malt extract, 7.5 g. For solid medium, 2% agar was added. Commercial potato-dextrose agar (PDA) was also used.

Salt solution contained NH_4NO_3, 24 g; KH_2PO_4, 16 g; Na_2SO_4, 4 g; KCl, 8 g; $MgSO_4 \cdot 7H_2O$, 2 g; $CaCl_2$, 1 g; trace element solution, 8 ml; and distilled water to 1 liter [after (5)].

Trace element solution contained H_3BO_3, 30 mg; $MnCl_2 \cdot 4H_2O$, 70 mg; $ZnCl_2$, 200 mg; $Na_2MoO_4 \cdot 2H_2O$, 20 mg; $FeCl_3 \cdot 6H_2O$, 50 mg; $CuSO_4 \cdot 5H_2O$, 200 mg; and distilled water to make 500 ml.

When minimal medium was buffered at pH values below 4 using a potassium phosphate (dibasic)—citric acid buffer (0.5 M), agar was autoclaved separately to prevent its hydrolysis. Individual growth factors were added as follows: vitamins, 1 mg/liter; purines and pyrimidines, 10 mg/liter; amino acids, 100 mg/liter.

Cultures were incubated at 25 C under constant fluorescent light, or at 30 C in the dark. Cultures stored on CM agar at 4 C remained viable for more than 1 year. Conidia of stock cultures were also preserved on silica gel (6) at 4 C, and most retained viability for over 2 years.

Biochemical and morphological mutants were isolated following irradiation of conidial suspensions at a dose killing 90 to 99% of the conidia. Heterokaryons were formed by placing mycelial inocula of complementary auxotrophs, or an auxotroph and a morphological mutant, together on MM. In the latter case, selection of morphologically wild-type growth through several successive transfers on MM was necessary.

Susceptible American chestnuts (*Castanea dentata* Borkh.) at least 1 inch in diam were inoculated in the field by a method suggested by R. A. Jaynes. A small disc of bark and underlying cambium was removed from the main stem of the tree using a cork-borer (8 mm diam) sterilized with 95% ethanol. A disc of mycelium cut out of an agar culture using the same borer was inserted in the hole in the bark. The hole was then sealed with tape. When two strains were inoculated into the same tree, the holes were made to touch. For laboratory inoculation, stems were cut in 6-inch segments, scrubbed with 20% commercial Clorox (NaClO, 5.25%) solution and 50% ethanol, and inoculated in

the same way as trees in the field. The segments were then placed upright in sterile, moist sand in closed plastic boxes or bottles and incubated at room temp near a window of the laboratory.

Stromata containing perithecia can be recognized by the many protruding dark perithecial necks. A rectangular cut was made in the bark containing these stromata, and the outer bark containing stromata and pycnidia was carefully peeled away from the underlying wood. Perithecia remaining embedded in the wood were then carefully dissected out, using sterile needles, and rolled over a hard agar surface to remove contaminating conidia. The perithecium was then crushed in a small amount of sterile distilled water (1 ml or less) and the suspension of asci was distributed over the agar. After 24 hr at 30 C, single germinated ascospores could be recognized using a magnification of ×90 on a stereo-dissecting microscope. The double-celled ascospores were easily distinguished from any single-celled conidia that were also present. Small pieces of agar, each with a single germinated spore, were cut out and transferred to fresh plates of CM, 16/plate.

Segregation of auxotrophic markers among ascospore progeny was tested on either MM or PDA with or without supplements. Auxotrophs will not produce conidia on PDA unless it is supplemented with the necessary growth factor. If a strain did not produce conidia on PDA after 5 days at 25 C, it was assumed to be an auxotroph. Sixteen different cultures could be tested on one plate of agar without interference from cross feeding.

RESULTS AND DISCUSSION.—*Nutrition.*—Natural isolates of *E. parasitica* and all derived strains produce abundant aerial, surface, and subsurface hyphae on CM. Pycnidia are formed along these hyphae, and those pycnidia exposed to the air exude their conidia in viscous drops. The hyphae are deep orange to brown; the pycnidia and conidia are orange-yellow. Vegetative transfers of natural isolates grow on MM but at a reduced rate, with thinner growth and little aerial mycelium. The yellow-orange pigment is usually restricted to the pycnidia and conidia which form profusely. If thiamine is missing, growth continues at an even slower rate and no pycnidia develop. The growth rate on PDA is comparable to that on CM but thinner, and aerial mycelium is reduced. Some pigment develops in the hyphae, but most is in the pycnidia and conidia. PDA supports the heaviest pycnidial production, and was used routinely for obtaining conidia.

Good growth and germination of conidia occurs on CM, but on PDA germination is erratic; often germlings do not develop into colonies.

Conidia form, but do not germinate on MM unless it is supplemented with 0.01% asparagine. Lower concn stimulate germination, but the germlings fail to develop into colonies. Aspartic acid, glutamic acid, and glutamine are also effective. A common biochemical interaction between glutamic acid and aspartic acid is a transamination in which the amino group is transferred, and amino nitrogen is probably the actual stimulator of germination. Biotin, which mediates aspartic acid synthesis, will also stimulate germination at concn as low as 10 μg/liter. Preformed mycelium does not have an absolute requirement for aspartate, and mycelium carried through three successive transfers on MM still grows. Additions of biotin or asparagine, however, enhance growth and pigmentation. Apparently, the mycelium can use inorganic nitrogen in the MM but not as effectively as the nitrogen in aspartate.

These results corroborate and clarify the findings of Bazzigher (2). Since in his nutrition studies he started with conidia, he could not separate those factors which stimulated spore germination from those which promoted hyphal growth. Our evidence suggests that spore germination and hyphal growth involve different metabolisms in *E. parasitica.* In particular, the need for biotin apparently decreases as the conidia germinate and develop. Similar differences have been reported in the fungus *Memnoniella echinata* by Perlman (7).

The optimal temp range of *E. parasitica* is 25 C to 30 C, but the fungus will grow at temp as low as 5 C and as high as 35 C. At 5 C, growth is extremely slow, and at 35 C it is slow and colony form is contorted.

The vegetative growth rate of *E. parasitica* is unaffected by light, but pycnidia will not form in the dark. Cultures on CM grown in the dark still develop the characteristic orange-brown pigment in their hyphae, but cultures on MM grown in the dark are colorless; such cultures on PDA are lightly pigmented. There is

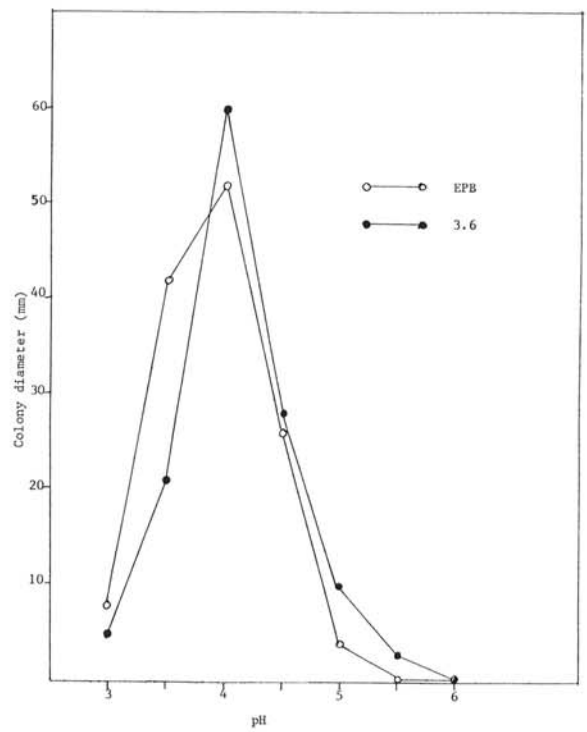

Fig. 1. Growth of two strains of *Endothia parasitica* at 30 C after 8 days on minimal medium agar buffered at various pH values with potassium phosphate (dibasic) and citric acid.

no evidence to suggest that the pigment in the hyphae is different from the pigment in the pycnidia.

The initial pH of unbuffered MM is about 4.5, and this value quickly drops with the growth of the fungus. Effect of pH on linear growth was estimated by growing the fungus on MM buffered at pH values from 3 to 7 (Fig. 1). At pH 5.5 or higher there is no growth. Best linear growth, at pH 4, was consistently better than growth on unbuffered MM. Wild-type strains will produce conidia on MM at all pH values which permit growth.

Induction and isolation of mutants.—The frequency of recovery of auxotrophic mutants ranged from 0.3 to 5%; the most useful mutants are: *col-1,* highly branched, thick, restricted growth; *col-2,* growth even more restricted than col-1; *cre-1,* light cream-colored conidia and mycelium; *ad-1,* adenine requiring; *arg-1,* arginine requiring, also responds to citrulline; *met-1,* methionine requiring, does not respond to homocysteine; and *rf-1,* riboflavin requiring. Most mutants were stable through frequent subculture and single conidial transfers. Conidia of arg-1, met-1, and ad-1 were tested for reversion to wild type, and none was found in over 4×10^6 conidia of each. The mycelia of most mutants are as long-lived as wild type, but mycelium of rf-1 dies very quickly even at 4 C. Conidia of the mutants show a variation in viability. Less than 10% of arg-1 spores germinate even when harvested from fresh cultures. The conidia of rf-1 and ad-1 died very quickly, even on silica gel. The rf-1 mutant and the ad-1 mutant were lost early in this study.

Morphological mutants produce pycnidia and conidia under the same conditions as wild-type strains. Biochemical mutants, however, produce conidia sparsely on CM and not at all on PDA. On both media, vegetative growth is slightly less than wild type. If the media are supplemented with the required biochemicals, the mutants will produce conidia profusely. Because all biochemical mutants so far tested show this phenomenon, the specific biochemical supplements are probably not involved directly in conidial production. Rather, the addition of the supplements may stimulate more vigorous growth which in turn triggers pycnidial development.

Several of the mutants were tested for their ability to infect chestnut trees and produce cankers bearing conidia. The mutants met-1, ad-1, and col-1 were as effective as wild type, and were therefore considered pathogenic. Mutants rf-1 and arg-1 were nonpathogenic.

Heterokaryosis.—The biochemical mutants ad-1, arg-1, rf-1, and met-1 were paired in all possible combinations on MM to synthesize heterokaryons. Subcultures of these heterokaryons on MM continued to show vigorous growth. Individual hyphal tips taken from some of them also gave rise to prototrophic cultures. The heterokaryons were stable on CM, and produced conidia on PDA which carried the markers of either one or the other of the parental pair.

Although heterokaryons can be readily obtained between two biochemical mutants, other evidence suggests that heterokaryosis is not common in this fungus. Under conditions where the heterokaryon is not strongly selected, e.g., between two morphological mutants or between a biochemical mutant and a morphological mutant, retrieval of the heterokaryon has not always been possible even after repeated attempts.

TABLE 1. Frequency of prototrophic conidial cultures from heterokaryons involving biochemical mutants of *Endothia parasitica*

Heterokaryon	Phenotypes of parents	Frequency of prototrophs
H1	Riboflavin requiring (rf-1) + arginine requiring (arg-1)	2.4×10^{-5}
H2	Methionine requiring (met-1) + arginine requiring (arg-1)	8.3×10^{-4}
H3	Methionine requiring (met-1) + adenine requiring (ad-1)	none among 1.3×10^7
H4	Methionine requiring (met-1) + riboflavin requiring (rf-1)	4.0×10^{-5}
H5	Arginine requiring (arg-1) + adenine requiring (ad-1)	none among 1.1×10^8

Because the heterokaryons between the various biochemical mutants are prototrophic, it follows that these mutants (rf-1, arg-1, ad-1, met-1) are recessive. The two morphological mutants, col-1 and col-2, which have been forced in heterokaryons are also recessive.

Parasexuality.—Since conidia of *E. parasitica* are almost always uninucleate, conidia of heterokaryons will give rise to colonies which are of either one parental type or the other. If the two parents are biochemical mutants, none of the conidia should grow on MM supplemented with asparagine. Any conidia that do grow would be heterokaryons (arising from rare binucleate spores), revertants, or diploids (resulting from a fusion of the two parental nuclei). Conidia from several heterokaryons were plated and the frequency of prototrophic colonies scored (Table 1). Not all heterokaryons yielded prototrophs, but in those that did, the frequency (1 to 4×10^{-5}) was higher than the rate of reversion ($< 4 \times 10^{-6}$). A prototroph from the heterokaryon No. 1 (H1) and one from heterokaryon No. 2 (H2) in Table 1 were studied in more detail. These prototrophs grew well on repeated subculture to MM, and also formed conidia on unsupplemented PDA. Conidia from both prototrophs were plated on MM with asparagine and all grew. Since all the conidia were thus prototrophic, the original isolates were not heterokaryons. It is likely, therefore, that these prototrophs are diploid. Further evidence that the prototroph of H1 is diploid is shown by irradiation of its conidia. Of 120 conidia which survived high doses of ultraviolet (99.9% kill), one required riboflavin. A second such irradiation yielded one which required arginine among 496 survivors. The phenotypes would be expected as the result of induced mitotic recombination in a heterozygous diploid (6).

Diploid formation in vegetative hyphae and subsequent mitotic recombination are two components of a parasexual cycle (9). Such a cycle permits genetic variability and recombination in a fungus without re-

TABLE 2. Phenotypes of colonies from ascospores resulting from artificial inoculations of chestnut trees with *Endothia parasitica*

Phenotypes inoculated	No. perithecia examined	Phenotypes of ascospore colonies & no. of each
Arginine requiring (arg-1), F[a]	2	60 wt (wild type)
Colonial morphology (col-1), F	4	25 wt; 37 colonial
Heterokaryon of arginine requiring (arg-1) + methionine requiring (met-1), L[b]	2	25 wt; 38 methionine requiring
Heterokaryon of arginine requiring (arg-1) + methionine requiring (progeny of heterokaryon met-1 + col-1 below), L	3	53 wt; 51 methionine requiring
Heterokaryon of methionine requiring (met-1) + colonial morphology (col-1), L	3	40 wt
	4	106 wt; 68 methionine requiring
	7	127 wt; 69 colonial
Heterokaryon of methionine requiring (met-1) + colonial morphology (col-1), F	1	32 methionine requiring (735 methionine requiring; 3 wt in a plating of the total contents of the perithecium)
	3	62 wt; 49 methionine requiring
	2	31 wt; 24 colonial; 23 methionine requiring
	1	5 wt; 3 colonial; 6 methionine requiring; 3 methionine requiring & colonial

[a] F = field inoculation.
[b] L = laboratory inoculation.

course to sexual mechanisms. Our evidence strongly suggests the existence of a parasexual cycle in *E. parasitica.*

Field and laboratory chestnut inoculations; analysis of sexual progeny.—Several inoculations of chestnuts both in the field and in the laboratory resulted in the development of perithecia and viable ascospores. This is the first report of successful inoculations in the laboratory. Most of the inocula carried genetic markers, and ascospores from individual perithecia were examined (Table 2).

The progenies from the heterokaryon between met-1 and col-1 (Table 2) are the most informative. Three of the perithecia from field inoculations produced asci among which segregation for both methionine requirement and colonial morphology was demonstrated. A fourth perithecium yielded only colonies which required methionine from 32 isolated ascospores. In addition, all 735 colonies arising from plating the entire contents of this perithecium were auxotrophs except for three wild-type colonies which were probably conidial contaminants. Since col-1 and met-1 arose from a common single conidial isolate, 3.6, they should be genetically identical except for the two marker mutations. These results therefore strongly suggest that a perithecium can arise from the sexual fusion of two genetically identical nuclei. In other words, there are no mating type loci in *E. parasitica;* it is homothallic.

Some complications are pointed up by the data for the remaining perithecia from the heterokaryon considered above, as well as the others listed in Table 2. Single-ascospore progeny from all three heterokaryons include wild-type strains which cannot be traced to the inoculum. Furthermore, only one of the two components of the heterokaryon was ever found in any one perithecium. It must be assumed that these perithecia arose from a fusion of one component of the heterokaryon with a wild-type contaminant. Since our chestnut material was collected from the field, such contamination is quite possible in spite of our precautions. These "crossed" perithecia are in the majority. Such high frequencies of crossed perithecia have also been observed in *Aspergillus nidulans* (9) and in *Glomerella cingulata* (10). Pontecorvo (8) has termed this "relative heterothallism"; that is, the fungus is homothallic but will outcross preferentially. The genetic basis for this is not known.

Endothia parasitica will not form perithecia or even perithecial initials on any of the agar media so far tested. These include CM, MM, PDA, malt agar, oatmeal agar, cornmeal agar, Wheeler's minimal *Glomerella* agar medium (10), and fresh chestnut bark autoclaved in 2% water agar. Wheeler et al. (10) showed, however, that in the closely related species *Glomerella cingulata,* the medium profoundly affects incidence of fruiting. We believe that eventually an artificial medium which supports perithecial formation will be found permitting more detailed studies of the genetics of *E. parasitica.*

LITERATURE CITED

1. ANDERSON, P. J. 1914. The morphology and life history of the chestnut blight fungus. Pa. Chestnut Tree Blight Commission Bull. No. 7.
2. BAZZIGHER, G. 1958. Der Wuchsstoffbedarf zweier phytopathogener Pilze. Phytopathol. Z. 32:352-358.
3. CAMPBELL, R. 1967. The interaction of carbon, nitrogen and sterilization of medium on pycnidial production of Endothia parasitica. Brit. Mycol. Soc. Trans. 50:413-421.
4. GYÖRGY, P. 1954. Biotin. *In* W. H. Sebrell, Jr., & R. S. Harris [eds.] The Vitamins Vol. I. Academic Press, N.Y.
5. HOLLIDAY, R. 1961. Induced mitotic crossing-over in Ustilago maydis. Genet. Res., Cambridge 2:231-248.
6. PERKINS, D. D. 1962. Preservation of Neurospora stock cultures with anhydrous silica gel. Can. J. Microbiol. 8:591-594.
7. PERLMAN, D. 1948. On the nutrition of Memnoniella

echinata and Stachybotrys atra. Amer. J. Bot. 35: 36-41.
8. PONTECORVO, G. 1958. Trends in genetic analysis. Columbia Univ. Press, N. Y. 145 p.
9. PONTECORVO, G., J. A. ROPER, L. M. HEMMONS, K. D. MACDONALD, & A. W. J. BUFTON. 1953. The genetics of Aspergillus nidulans. Advanc. Genet. 5:141-238.
10. WHEELER, H. E., C. H. DRIVER, & C. CAMPA. 1959. Cross- and self-fertilization in Glomerella. Amer. J. Bot. 46:361-365.