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Formation of perithecia of Ceratocystis ulmi on natural and synthetic nutrient media[1]

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Abstract

Ceratocystis ulmi formed fertile perithecia on 17 of 37 culture media tested, including many of the (autoclaved) natural substrates, but not on Saboraud's media or White's medium and not on potato dextrose agar (Table 1). Later, perithecia formed abundantly on four of eight variants of Zentmyer-Tchernoff agar medium (Table 2). This appears to be the first report on perithecia of *C. ulmi* on an agar medium to which only synthetic nutrients were added. Both normal-strength and 1:10 diluted media gave perithecia. If only the sugar or only the asparagine-plus-salts were diluted, none or very few perithecia formed. Tenfold dilution of the vitamins did not affect results. Light did not prevent formation of perithecia.

Introduction

Since Buisman (1932) first described production of perithecia of "*Ceratostomella*" *ulmi* (re-named *Ceratocystis ulmi* (Buism.) C. Moreau in 1952) in culture by mating two isolates of *Graphium ulmi* Schwarz on sterilized elm wood, investigators have used this natural substrate to study the ascocarp of this fungus (Shafer and Liming, 1950; Rosinski, 1958 and 1961; Holmes and Demaradzki, 1957 and 1959; Holmes, 1958 and 1965). Perithecia of *C. ulmi* have not been reported on synthetic media.

Graphium ulmi grows well on agar media, which are easier to prepare in quantity than wood substrates. If perithecia could be produced on a medium whose nutrient content is fully known, one could study the influence of particular nutrients and of nutrient balance upon the production of perithecia.

Materials and methods

Media

1. In the first series, "A" and "B" strains of *C. ulmi* were crossed by adjacent inoculation onto the Difco potato dextrose agar used regularly in Control Service isolations from diseased elm specimens submitted to the Shade Tree Laboratories.

2. Crosses in the second series were made onto autoclaved plant or animal products, some with and some without agar, as listed in Table 1, and on White's tissue-culture agar medium and on Saboraud's agar media. These culture media form part of the standard collection of media of the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

[1] A contribution of the Massachusetts Agricultural Experiment Station.

Table 1. Formation of perithecia of *Ceratocystis ulmi* on various culture media inoculated once with compatibility type A and once with B in any of four mating combinations (the first number in each is A).

Medium	Mycelial growth	Coremia	Perithecia from cross between:			
			NL-1 × TX21-4	NL-1 × NL-6	TX50-1 × NL-6	TX50-1 × TX21-4
Tree tissues						
Unsplit peeled elm twigs	heavy	+	many	many	0	sev.
Split peeled elm twigs	heavy	+	many	many	0	sev.
Elm twigs with bark	heavy	+	few	many	2(def.)	many
Apple twigs	heavy	+	0	0	0	many
Maple twigs	moderate	+	many	many	sev./many	many
Pine twigs	moderate	+	0	1?(im.)	0	0
Poplar twigs	moderate	0	0	0	0*	0*
Spruce twigs	moderate	+	0	0	0	few
Willow twigs	moderate	+	0	0	0	3(im.)
Alder twigs	heavy	+	0	0	0	0*
Oak twigs	heavy	+	0	many	0	many
Non-tree plant tissues						
Tomato stems	moderate	+	0	0	0	0
Rye spikes	heavy	0	0	0*	0	0
Lupine stems	moderate	+	sev.	0	0	0
Potato tuber pieces/glycerine	heavy	+	0	sev./many	0	sev.
Potato tuber pieces	heavy	+	0	0	0	0
Carrot root pieces	heavy	0	0	sev./many	0	0
Hyacinth bulb pieces	moderate	0	0	0	0	0
Barley corns (seeds)	heavy	0	0*	sev.(im.)	0	0*
Rice (seeds)	heavy	0	0	0	0	0
Non-plant substances						
Earth**	moderate	+	0***	0***	0***	0***
Clay	sl./mod.	+	0***	0***	0***	0***
Rabbit dung	slight	0	0	0	0	0
Agar media with plant substances						
Malt agar	heavy	+	0	many	0	few (2)
20% malt agar	heavy	+	0	sev./many	0	sev. (6)
Malt agar, 40% sugar	slight	0	0	0	0	0
Malt-salep agar	heavy	+	0	0	0	0
Cornmeal agar	heavy	+	0	many	0	0
Oatmeal agar	heavy	+	few	many	0	0
Oatmeal alkaline agar	heavy	+	sev./many	sev./many	sev.	sev.
Oatmeal-dung agar	moderate	+	few	very many	0	0
Cherry fruit-extract agar	heavy	+	8(im.)	few (1)	0	0
Hay agar	slight	+	0	0	0	0
Potato agar	slight	0	0	0	0	0
Agar media with inorganic salts						
Saboraud glucose agar	heavy	+	0	0	0	0
Saboraud maltose agar	heavy	+	0	0	0	0
White's tissue culture agar	scanty	0	0	0	0	0

* Inoculum failed to grow in this cross.

**Apparently sand plus humus, bits of bark, broken twigs and peanut shells.

*** Difficult to observe; perithecia might have been overlooked in the clutter.

Few = 1–5; sev. = 6–25; many = 26 or more; im. = immature; def. = deformed or distorted.

Tabel 1. Vorming van peritheciën van Ceratocystis ulmi op verschillende voedingsmedia. Ieder medium werd in elk der vier mogelijke sexuele combinaties éénmaal geïnoculeerd met compatibiliteitstype A en éénmaal met type B (type A is steeds als eerste vermeld).

Table 2. Formation of perithecia of *Ceratocystis ulmi* on culture media containing glucose, asparagine, KH_2PO_4, $MgSO_4.7H_2O$, $ZnSO_4$, $FeCl_3$, pyridoxine, thiamine, and agar (Tchernoff, 1965), inoculated 31-1-1969.

Formula	Container	Replicates	Number of replicates with perithecia on date:		
			14-2-1969	10-3-1969	24-3-1969
H-H-H	Petri dishes	5	0	0	*
H-H-H	tubes, incubator	10	1	4	4
H-H-H	tubes, table	10	2	5	5
L-H-H	Petri dishes	5	0	0	*
L-H-H	tubes, incubator	10	0	0	0
L-H-H	tubes, table	10	0	0	0
H-L-H	Petri dishes	5	0	0	*
H-L-H	tubes, incubator	10	0**	0	0
H-L-H	tubes, table	10	0**	0	1***
H-H-L	Petri dishes	5	1	2	*
H-H-L	tubes, incubator	10	5	6	6
H-H-L	tubes, table	10	0	3	3
L-L-H	Petri dishes	5	0	0	*
L-L-H	tubes, incubator	10	2	3	3
L-L-H	tubes, table	10	1	2	2
L-H-L	Petri dishes	5	0	0	*
L-H-L	tubes, incubator	10	0	0	0
L-H-L	tubes, table	10	0	0	0
H-L-L	Petri dishes	5	0	0	*
H-L-L	tubes, incubator	10	0	0	0
H-L-L	tubes, table	10	0	0	0
L-L-L	Petri dishes	5	0	0	*
L-L-L	tubes, incubator	10	3	3	3
L-L-L	tubes, table	10	4	4	4

* Petri dishes were discarded after the second reading because they had dried out.

** On the H-L-H medium, deformed coremia developed.

*** A single perithecium, the only one in a medium with HL or LH sugar-salt ratio.

H = high (1 × the amount in Tchernoff's medium); L = low (0.1 ×). In the formula, the first letter is for sugar, the second for salts, and the last for vitamins.

Tabel 2. Vorming van peritheciën van Ceratocystis ulmi op een voedingsmedium dat glucose, asparagine, KH_2PO_4, $MgSO_4.7H_2O$, $ZnSO_4$, $FeCl_3$, pyridoxine, thiamine en agar bevat (Tchernoff, 1965). Inoculatiedatum: 31-1-1969.

3. Compatible crosses of the third series were made on eight variants of Zentmyer-Tchernoff medium (Tchernoff, 1965), as itemized in Table 2. These eight media contained, per liter, (a) of sugar: either 20 g or 2 g glucose, (b) of salts: either 2 g asparagine + 1.5 g KH_2PO_4 + 1 g $MgSO_4.7H_2O$ + 20 mg $ZnSO_4$ + 10 mg $FeCl_3$ or one-tenth of these amounts, respectively, and (c) of vitamins: either 1 mg or 0.1 mg of each of pyridoxine (B_6) and thiamine (B_1). All eight media contained 15 g agar per liter. These media were named by a 3-letter code, where the first letter stood for sugar, the second letter for asparagine and salts, and the last letter for vitamins; "H" meant the higher concentration (1 × the amount in Tchernoff's medium) and "L" meant the lower concentration (0.1 ×).

Perithecial production on all media was compared to that on moist, autoclaved elm wood slices or peeled elm twigs.

Inocula

1. In the first series, the cultures mated were isolated from *Ulmus americana* L. in Carlisle, Mass. (compatibility type A) and in Marshfield, Mass. (type B).

2. In the second series, the cultures mated were numbers NL-1 (compatibility type A) and NL-6 (type B), both isolated by the author from twigs of *Ulmus hollandica* Mill. cl. 'Belgica' collected in Naarden, The Netherlands, and TX50-1 (compatibility type A) from Barsingerhorn, The Netherlands, and TX21-4 (type B) from Baarn, The Netherlands, furnished by Ir V. Tchernoff (Tchernoff, 1965).

3. In the third series, the cultures mated were numbers 68.12082 (compatibility type A) from Amherst, Mass., and 68.12018 (Type B), from Newton, Mass., both isolated from diseased trees of *U. americana* in December 1968.

Replication and incubation

1. Crosses in the first series were made in ten test tubes and in ten Petri dishes. The cultures then were kept in the dark at 25 °C or on the laboratory table at room temperature.

2. Crosses in the second series were made in two test tubes for each of the four combinations of A × B cultures for each of the 37 media, resulting in eight crosses per medium. These tubes were incubated in the dark at 25 °C. The results were observed 90 days after inoculation.

3. In the third series, each cross was replicated in 20 test tubes and five Petri dishes for each of the eight media. Ten tubes of each were incubated in the dark at 25 °C for 11 days and then placed on the laboratory table at normal day-night variations of light and temperature. The other ten tubes and the five Petri dishes were left on the table throughout the experiment. Results of these crosses were observed 14, 38, and 52 days after inoculation.

Results

1. No perithecia formed on the potato dextrose agar in any series, but perithecia formed on slices of peeled elm branches or split elm twigs in all series.

2. Perithecia extruding ascospores formed on 17 of the 37 media in the second series, and immature perithecia were found on three other media (Table 1). However, on only three media did all four of the different crosses of A × B (NL-1 × TX21-4, NL-1 × NL-6, TX50-1 × TX21-4, and TX50-1 × NL-6) yield perithecia. These substrates were: maple twigs, elm twigs, and alkaline oatmeal agar. No perithecia formed on agar media that contained only known chemicals.

3. Perithecia extruding ascospores formed sparsely in two Petri dishes and abundantly in 30 test tubes out of the 200 trials in the third series (Table 2), but on only four of the eight media, namely HHH, HHL, LLH, and LLL. No perithecia had formed on media that combined high sugar with low salts or vice versa (LHH, HLH, LHL, HLL) by the 14th or the 38th day after inoculation. However, on the 52nd day, when the tubes were given a final examination before discarding, one perithecium was found in one test tube containing the HLH medium. In no case (Table 2) did perithecia occur in more than 6 of the 10 tubes of a given treatment. Often, when they occurred at all, it was in no more than 3 of the 10 tubes.

Discussion

Perithecia formed in some of the crosses on a given medium in the second series but were not found on the same medium in other crosses. They might have been found if there had been more replicates, since in the third series they never formed in more than 60% of the tubes.

In the third series, when perithecia formed at all, they usually appeared profusely. Yet in the same treatment some tubes could always be found in which no perithecia occurred. This "all or none" phenomenon might be interpreted as a failure of gametangial contact to take place, an event necessary for ascocarp formation.

The difference between the higher level and the lower level of vitamins in the Zentmyer-Tchernoff medium did not affect production of perithecia. However the balance between concentrations of sugar and asparagine-plus-salts appeared to be critical.

Humidity, depth of medium, toxic excretory products of growth, aeration, mixing of inoculum during inoculation, all differ between test tubes and Petri dishes. Which of these factors or what combination of factors have a bearing on perithecial formation is not known. Depth of medium could affect downward diffusion of toxic excretory byproducts of vegetative growth. Inocula tend to mix at early stages of growth on the slanting surfaces of the agar in the test tubes.

The formation of few or no perithecia by the cross TX50-1 × TX21-4 in the second series may be the result of prolonged maintenance in culture. Over a period of 13 years it has repeatedly been found that the cultures used as standards for types A and B, in crosses to determine compatibility of newly isolated cultures, gradually lose their ability to form perithecia even under the most favorable circumstances of media and incubation. New standard cultures have had to be set up every third or fourth year, and their mating types determined by crossing with the former standard cultures.

The terminology A and B for the cultures in this work is the same as that used by Shafer and Liming (1950). Efforts to correlate with the + and − terminology of Buisman were not successful, as no cultures were found that had been isolated by her or had been typed by crossing with hers, and the two compatibility types are found throughout the area from which she obtained her isolates.

The availability of a technique for production of perithecia of *Ceratocystis ulmi* on a medium that contains only synthetic nutrients opens the vista of study of the nutrient requirements for formation of the sexual stage of this fungus. Further studies, however, are needed to eliminate the possibility that the agar or the Pyrex glass may be contributing one or more essential micro-nutrients.

In future studies of relative nutrient levels, the nitrogen source needs to be varied independently of the major source of carbon and of the salts.

The suitability of a culture medium that can be processed rapidly and easily in large quantities, and one that is best adapted to test tubes, should facilitate study of inheritance and variation in this fungus.

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Samenvatting

Vorming van peritheciën van Ceratocystis ulmi op natuurlijke en synthetische voedingsmedia

Ceratocystis ulmi vormde rijpe peritheciën op 17 van de 37 getoetste voedingsbodems, maar in het begin alleen op enkele van de (gesteriliseerde) natuurlijke voedingsbodems, niet op Sabourauds of Whites agar en evenmin op aardappel-dextrose-agar (Tabel 1). In latere proeven werden rijkelijk peritheciën gevormd op 4 van de 8 varianten op Zentmyer-Tchernoff-agar (Tabel 2).

Waarschijnlijk is dit de eerste vermelding van vorming van peritheciën van *C. Ulmi* op agar waaraan uitsluitend synthetische voedingsstoffen zijn toegevoegd. Zowel op de standaard-concentratie als op een tienvoudige verdunning van de voedingsstoffen werden peritheciën gevormd. Als alleen de glucose of alleen de asparagine-plus-zouten verdund waren, werden er geen of zeer weinig peritheciën gevormd. Tienvoudige verdunning van de vitaminen beïnvloedde de resultaten niet. Licht remde de vorming van peritheciën niet.

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[1] Photocopy in English translation available from F. W. Holmes, Shade Tree Laboratories, University of Massachusetts, Amherst, Mass., 01002, U.S.A., at current cost.