Production of Synnemata on Defined Agar Media and Its Relation to Pathogenicity of Ceratocystis ulmi

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ABSTRACT

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Defined agar media were developed which were suitable for synnema production by some isolates of *Ceratocystis ulmi*. Several media supported synnema production, but the best medium contained (per liter): 2.0 g glucose, 0.08 g L-asparagine, macro- and microelements, pyridoxine, and 0.50 g linoleic acid. Production of synnemata on this medium varied among isolates of *C. ulmi* with known pathogenicities, but more aggressive isolates generally produced more synnemata than less aggressive ones.

Additional key words: stearic acid, oleic acid, C:N ratio.

Variation in cultural characteristics and pathogenicity of *Ceratocystis ulmi* (Buism.) C. Moreau., the Dutch elm disease fungus, has been reported (4, 16, 19, 28). Recently, in Great Britain (2, 7, 8) and the United States (25, 26), variation in culture has been associated with variation in pathogenicity. The more aggressive isolates of *C. ulmi* generally grew faster radially on agar media than did less aggressive ones (2, 7, 8, 25, 26). The more aggressive isolates from Great Britain also produced more aerial mycelium on a malt extract agar medium than did less aggressive ones (2, 7, 8), a relationship not observed with all isolates from the U.S. (8, 25, 26). Generally, more aggressive isolates from the U.S. also produced more synnemata on wood disks of *Ulmus americana* L. and *U. pumila* L. than did less aggressive ones (25, 26). Because of this relationship, our efforts were directed to the development of a standard agar medium suitable for studying variation in synnema production among isolates of *C. ulmi*.

Most isolates of *C. ulmi* do not produce synnemata on agar media (18) unless elm wood pieces or elm wood extracts are included (5, 13, 18, 19, 21, 27). Extracts of elm wood, including a water-soluble fraction containing various nutrients (18, 19, 21, 27), a chloroform-soluble fraction containing linoleic acid and its esters as active components (18, 19, 20, 21), and a phenolic fraction (27) are reported to be important for synnema formation by these isolates. However, three isolates with darkly pigmented mycelia have been reported to produce synnemata on agar media not containing elm wood pieces or extracts (1, 10, 11, 12, 29). One isolate produced synnemata on defined agar media and on a potato-dextrose agar medium (10, 11, 12). Another isolate produced synnemata on malt extract agar medium (29), and media containing oleic acid enhanced synnema formation by the third isolate (1). Recently, a Canadian isolate produced synnemata on a potato-dextrose agar medium containing certain terpenes and unsaturated fatty acids, particularly linoleic acid (17).

The purpose of this report is to describe the development of media that support synnema production by an isolate of *C. ulmi,* and to present evidence that synnema production on a defined agar medium is variable and related to pathogenicity in *C. ulmi*. A preliminary report on a portion of this work has been published (14).

MATERIALS AND METHODS

The pathogenicity of all *C. ulmi* isolates used in these studies was determined previously by inoculation trials (8, 25, 26). Twelve isolates were supplied by L. R. Schreiber, USDA-ARS, Shade Tree and Ornamental Plants Laboratory, Delaware, OH 43015. Seven isolates were supplied by H. S. McNabb, Jr., Iowa State University, Ames, IA 50010 (Table 1). For clarity, only two categories: "more aggressive" and "less aggressive", will be used to describe degree of pathogenicity. "More aggressive" will be used for all isolates previously described as "more aggressive" and "aggressive". "Less aggressive" will be used for all isolates previously described as "less aggressive", "nonaggressive", or with "intermediate aggressiveness" (8, 26).

All isolates were maintained in screw-top test tubes on a glucose-yeast extract agar medium at 5 C (23). Before they were transferred to experimental media, isolates were incubated on the maintenance medium (23) for 5 to 7 days at 25 C. Mycelium from the advancing margin of

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these cultures was transferred to experimental media and incubated at 25 C for 14 days in diffuse light or total darkness. Synnema production was rated with a binocular microscope as follows: 1 = none; 2 = rare; 3 = few; and 4 = abundant. Five replications of each treatment were used in each experiment.

A basal medium which did not support synnema production by any of the isolates of *C. ulmi* used in these studies (Table 1) was modified and tested for synnema production. The basal medium contained 10.0 g glucose, 2.0 g L-asparagine, 1.0 g KH_2PO_4, 0.50 g $MgSO_4 \cdot 7H_2O$, 0.20 mg Fe^{++}, 0.20 mg Zn^{++}, 0.10 mg Mn^{++}, 0.10 mg pyridoxine, 15.0 g Difco Bacto agar, and 1,000 ml distilled water (23). This medium was modified by changing the concentrations of glucose and L-asparagine and adding one of three 18-carbon fatty acids: stearic acid (18:0) (Fisher, reagent grade); oleic acid (18:1) (Fisher, purified); and linoleic acid (18:2) (Fisher, purified). Fatty acids were added to the media after other components were dissolved and pH was adjusted to 6.0 ± 0.1 with 1N KOH or 1N H_2SO_4 before autoclaving. Details regarding media composition are given under specific experiments.

RESULTS

To determine whether isolate WIS-1 (Table 1) would produce synnemata on modified basal medium containing 0.50 g/liter oleic acid, the concentrations of glucose and the ratios of glucose to L-asparagine were varied (1). Four concentrations of glucose: 10.0, 5.0, 2.0, and 1.0 g/liter and five glucose to L-asparagine ratios: (1 to 1, 5 to 1, 10 to 1, 25 to 1, and 50 to 1) were used (Fig. 1).

Limited numbers of synnemata were produced on some of these media (Fig. 1). Synnema production was favored on media with low concentrations of glucose and high glucose to L-asparagine ratios. The medium containing 2.0 g glucose with a 25 to 1 glucose to L-asparagine ratio

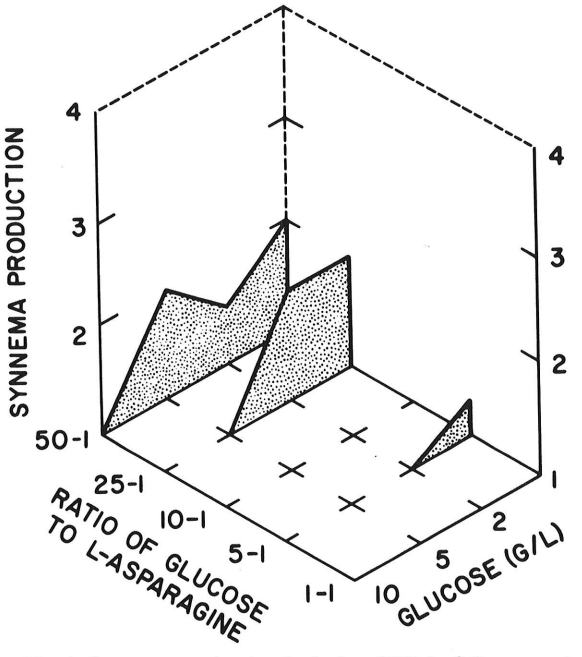

Fig. 1. Synnema production by isolate WIS-1 of *Ceratocystis ulmi* grown on modified basal media containing 0.50 g/liter oleic acid.

TABLE 1. Origin and pathogenicity of isolates of *Ceratocystis ulmi* used to develop agar media for synnema production

Origin	Code	Pathogenicity	Ref.[a]
Alabama[b]	ALA-1	More aggressive	(26)
Colorado[b]	COLO-1	More aggressive	(26)
Great Britain[c]	GB-1	More aggressive	(8)
Great Britain[c]	GB-2	More aggressive	(8)
Illinois[b]	ILL-1	More aggressive	(26)
Iowa	IA-1	More aggressive	(8)
Massachusetts[b, d]	MASS-1	More aggressive	(26)
Missouri[b]	MO-1	More aggressive	(26)
North Dakota[b]	ND-1	More aggressive	(26)
Wisconsin[b]	WIS-1	More aggressive	(26)
Great Britain[c]	GB-3	Less aggressive	(8)
Great Britain[c]	GB-4	Less aggressive	(8)
Iowa[c]	IA-2	Less aggressive	(8)
Iowa[c]	IA-3	Less aggressive	(8)
Massachusetts[b]	MASS-2	Less aggressive	(26)
Maine[b]	ME-1	Less aggressive	(26)
North Carolina[b]	NC-1	Less aggressive	(26)
Ohio[b]	OH-1	Less aggressive	(26)
Tennessee[b]	TENN-1	Less aggressive	(26)

[a]References refer to work determining isolate pathogenicity.
[b]Isolates supplied by L. R. Schreiber.
[c]Isolates supplied by H. S. McNabb, Jr.
[d]This isolate was shown to be less aggressive by Schreiber and Townsend (26), but results from subsequent inoculation trials show our culture of this isolate to be more aggressive (15).

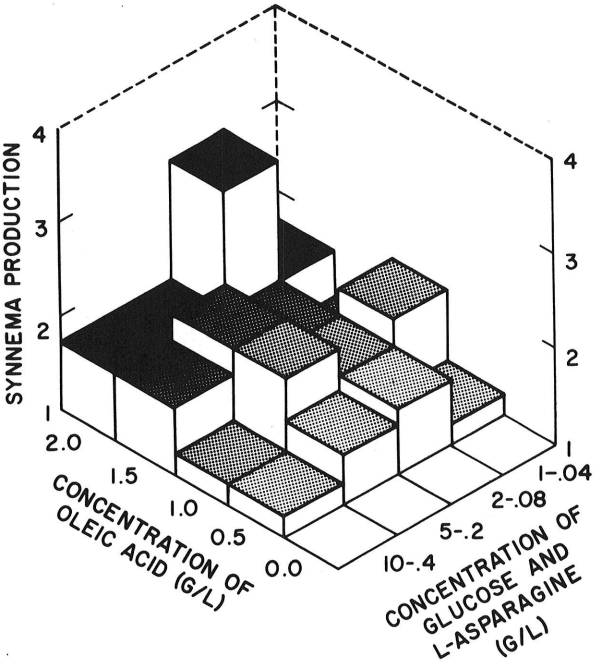

Fig. 2. Synnema production by isolate WIS-1 of *Ceratocystis ulmi* grown on modified basal media containing five levels of oleic acid.

was favorable for synnema production. Further reduction in glucose concentrations or increases in the glucose to L-asparagine ratio did not enhance synnema production (Fig. 1).

Based on these results, additional studies were initiated to test the affect of five concentrations of oleic and linoleic acid in media containing: 10.0 and 0.40; 5.0 and 0.20; 2.0 and 0.80; and 1.0 and 0.04 g/liter glucose and L-asparagine, respectively (1, 17, 18, 20). To each of these media were added (per liter): 0.00, 0.50, 1.00, 1.50, or 2.00 g oleic or linoleic acid (Fig. 2 and 3).

No synnemata were produced on media not containing oleic acid or linoleic acid (Fig. 2, 3). With one exception, only limited synnema production occurred on media containing oleic acid (Fig. 2). When linoleic acid was present, abundant synnema production occurred on all media containing low concentrations of glucose and L-asparagine. These results indicated that a modified basal medium containing 2.0 g glucose, 0.08 g L-asparagine and 0.50 g linoleic acid was very favorable for synnema production (Fig. 3). In two additional studies, this medium and several modifications of it were used to test the synnema production capabilities of all isolates of *C. ulmi* of known pathogenicity in our culture collection (Table 1).

The first of these studies tested the affect on synnema production of 0.00, 0.01, 0.10, 0.25, and 0.50 g/liter linoleic acid (Table 2). Synnema production by isolates of known pathogenicity was variable on these media, but no synnemata were produced on media containing 0.00 or 0.01 g linoleic acid. In general, the medium containing 0.50 g linoleic acid was most favorable for synnema production, and the more aggressive isolates produced more synnemata on this medium than did less aggressive ones (Table 2).

The second study tested the affect on synnema production of two additional 18-carbon fatty acids. One-half gram of stearic acid or oleic acid were substituted for 0.50 g linoleic acid in the medium. (Table 3). No synnemata were produced on the medium without fatty acids, and with one exception, no synnemata were produced on the medium containing stearic acid. The medium containing oleic acid supported some synnema production, but the largest number of synnemata were produced on the medium containing linoleic acid. More aggressive isolates generally produced more synnemata on this medium than did less aggressive ones (Table 3).

DISCUSSION

By modifying the constituents of a defined agar medium (23), we have developed media, not containing elm wood pieces or extracts, that supported synnema production by all of the more aggressive isolates of *C. ulmi* tested and only a few of the less aggressive ones (Fig. 2, 3 and Tables 2, 3). Optimal synnema production generally occurred on media containing linoleic acid with reduced concentrations of glucose and high glucose to L-asparagine ratios. Of all media tested, greatest numbers of synnemata were produced on media containing 2.0 g glucose, 0.08 g L-asparagine, and 0.50 g linoleic acid per

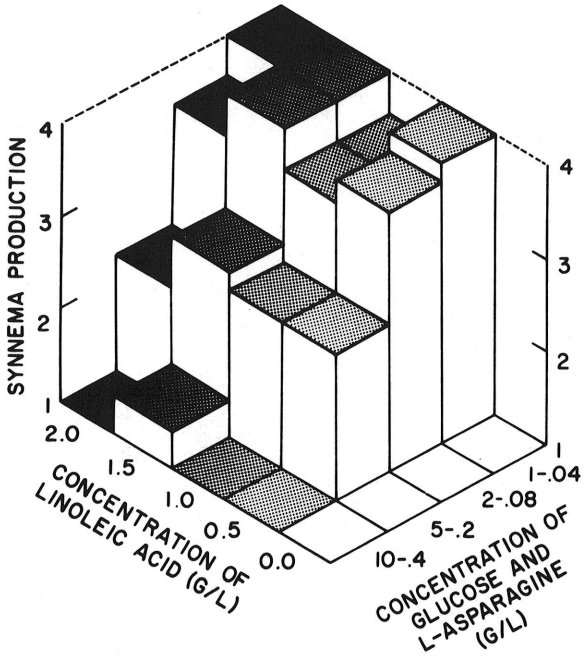

Fig. 3. Synnema production by the isolate WIS-1 of *Ceratocystis ulmi* grown on modified basal medium containing five levels of linoleic acid.

TABLE 2. Synnema production by isolates of *Ceratocystis ulmi* grown on modified basal medium containing 2.0 g glucose, 0.08 g L-asparagine, and several concentrations of linoleic acid (per liter)

Isolates	Concentration of linoleic acid				
	0.00	0.01	0.10	0.25	0.50
More aggressive					
ALA-1	1.0[a]	1.0	3.0	4.0	4.0
COLO-1	1.0	1.0	3.0	4.0	4.0
GB-1	1.0	1.0	1.6	3.0	4.0
GB-2	1.0	1.0	2.0	4.0	4.0
ILL-1	1.0	1.0	1.0	1.0	1.4
IA-1	1.0	1.0	2.0	3.0	4.0
MASS-1	1.0	1.0	3.8	4.0	4.0
MO-1	1.0	1.0	1.0	1.0	1.3
ND-1	1.0	1.0	3.0	4.0	4.0
WIS-1	1.0	1.0	3.0	4.0	4.0
Mean synnema production	1.0	1.0	2.3	3.2	3.5
Less aggressive					
GB-3	1.0	1.0	1.0	1.2	1.0
GB-4	1.0	1.0	1.0	1.0	1.2
IA-2	1.0	1.0	1.0	1.0	1.0
IA-3	1.0	1.0	1.0	1.0	1.0
ME-1	1.0	1.0	1.0	2.4	3.4
MASS-2	1.0	1.0	1.0	1.0	1.0
NC-1	1.0	1.0	1.0	1.0	1.0
OH-1	1.0	1.0	4.0	4.0	4.0
TENN-1	1.0	1.0	1.0	1.0	1.0
Mean synnema production	1.0	1.0	1.4	1.5	1.7

[a]Synnema production ratings: 1 = none; 2 = rare; 3 = few; 4 = abundant.

liter. This medium also favored pigment formation in those isolates producing synnemata and will be referred to as synnema production medium (SPM).

The SPM medium provides only 1.215 g/liter carbon and 0.017 g/liter nitrogen. Thus, it has relatively low levels of carbon and a high carbon-to-nitrogen (C:N) ratio: 71.5 : 1. The nutrient availability and C:N ratio of SPM may be similar to those of elm wood. Wood is generally considered to have a high C:N ratio because it contains large amounts of carbonaceous materials, mainly cellulose, and small amounts of nitrogenous materials (3). A report indicates that elm branch wood may have C:N ratios as high as 26:1 (24). In addition, SPM and elm wood contain linoleic acid (19, 20). Linoleic acid was required in SPM for best synnema production by most isolates of *C. ulmi* tested (Tables 2, 3), and best synnema production occurred on elm sapwood, the area in elm wood containing the most linoleic acid (19, 20). But a blue stain fungus, *Ceratocystis piceae* (Munch) Bakshi, produced synnema on a medium with a low level of nitrogen when exposed to gaseous aliphatic aldehydes (6). Since these materials are known to be breakdown products of fatty acids including linoleic, it was suggested that aliphatic aldehydes might be the active component in elm wood that triggers synnema production by *C. ulmi* (6). However, no aldehydes have been found in elm wood (20) and, although the possibility that breakdown products of linoleic acid might have had some effect on synnema production on SPM, the combination of low levels of nutrients, high C:N ratio and linoleic acid required for best synnema production on agar media may be similar to the nutrient complement in elm wood.

We have shown that isolates of *C. ulmi* differ in capabilities for synnema production on SPM. Generally, the more aggressive isolates produced more synnemata on SPM than did the less aggressive ones. A similar relation between synnema production on elm wood disks and pathogenicity has been reported by Schreiber and Townsend (25, 26). Since twelve of the isolates (Table 1) used in our studies also were used by Schreiber and Townsend (25, 26), our results further substantiate their conclusions. Nevertheless, several isolates did not follow the general pattern. Two of the more aggressive isolates, ILL-1 and MO-1, and one less aggressive one, ME-1, produced numbers of synnemata that were intermediate between the more- and the less-aggressive isolates. The intermediate synnema production capability of ME-1 is especially interesting since this isolate also has been reported to have an intermediate level of pathogenicity (26). The less aggressive isolate OH-1 produced abundant synnemata on several media that were not favorable for synnema production by other isolates. The significance of the synnema production capabilities of this isolate is not known, but our results suggest that there may be more variability among isolates of *C. ulmi* than some recent studies have indicated (2, 8).

Production of synnemata in beetle galleries is important for effective beetle transmission of *C. ulmi*. The ability of an isolate to produce synnemata in beetle galleries would enhance the probability that it could be spread effectively by beetles. More aggressive isolates are the most common in North America (2) and are rapidly spreading across Great Britain (9). Since this type of isolate spreads so effectively and produces more synnemata on elm wood disks (25, 26) and on SPM than the less aggressive isolates, more study on the synnema production capabilities among isolates of *C. ulmi* is needed. With the development of a defined agar medium that seems to be suitable for studying synnema production in vitro, new studies on the physiological basis for synnema production and its relation to pathogenicity now are possible.

TABLE 3. Synnema production by isolates of *Ceratocystis ulmi* grown on modified basal medium containing 2.0 g glucose, 0.08 g L-asparagine and 0.50 g steric, oleic, or linoleic acid (per liter)

Isolates	Fatty acids			
	None	Stearic	Oleic	Linoleic
More aggressive				
ALA-1	1.0[a]	1.0	2.0	4.0
COLO-1	1.0	1.0	2.4	4.0
GB-1	1.0	1.0	3.0	4.0
GB-2	1.0	1.2	2.6	4.0
ILL-1	1.0	1.0	1.0	2.6
IA-1	1.0	1.0	2.0	4.0
MASS-1	1.0	1.0	3.3	4.0
MO-1	1.0	1.0	1.0	3.0
ND-1	1.0	1.0	2.0	4.0
WIS-1	1.0	1.0	3.0	4.0
Mean synnema production	1.0	1.0	2.2	3.8
Less aggressive				
GB-3	1.0	1.0	1.0	2.2
GB-4	1.0	1.0	1.0	1.6
IA-2	1.0	1.0	1.0	1.0
IA-3	1.0	1.0	1.0	1.0
ME-1	1.0	1.0	1.0	2.2
MASS-2	1.0	1.0	1.0	1.0
NC-1	1.0	1.0	1.0	1.0
OH-1	1.0	1.0	4.0	4.0
TENN-1	1.0	1.0	1.0	1.0
Mean synnema production	1.0	1.0	1.4	1.8

[a]Synnema production ratings: 1 = none; 2 = rare; 3 = few; 4 = abundant.

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