Studies on the Biology of Gnomonia fragariae Klebahn[1]

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Thirty-eight monoconidial isolates of *Gnomonia fragariae* comprised at least 6 races that differed in one or more of the following characters: appearance of colonies on artificial media, nutritional requirements for growth and sporulation, temperature relations, relation to hydrogen-ion concentration, and sporulating ability. At least six distinct races were isolated from a single pycnidium on a leaf of wild strawberry.

None of the 4 races studied intensively for nutritional requirements grew well on a basal synthetic medium. When vitamins were added to the basal solution the different races grew well, but the vitamin requirements differed for each of the four races. Temperature requirements differed for the different races. Four were studied intensively, and the optimum temperatures for two of them was 25°C., while that for the other two was 20°C. In general, the different races studied grew somewhat at a range of hydrogen-ion concentration from 3.2 to 9.1. Of the four races studied thoroughly, however, the optimum for one race was 4.7, that for two races was 5.5 and that for the fourth race was 6.2.

Of the total of 38 isolates studied, 33 produced parithecia, while only 5 produced both perithecia and pycnidia. These isolates produced perithecia in both monoconidial and single-ascopore cultures. The best medium for sporulation for all of the races was cornmeal-infusion-malt agar. This medium apparently contains substances that could not be supplied to most races by means of any of the nine vitamins tried. The specific vitamin requirements, either ascorbic acid, pyridixine, or inositol were determined for only one race.

INTRODUCTION

Gnomonia fragariae Klebahn was discovered on strawberry petioles by Klebahn near Hamburg, Germany, in 1908. In the United States, the fungus was first reported by Alexopoulos and Cation (1948), on the calyces and

1. The work was done while the author was a graduate student at the University of Minnesota as a scholar of the Central Government of Pakistan.

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peduncles of strawberries and on maltose and cornmeal agar. The perithecial stage was assigned to *Gnomonia* at that time and has recently been identified by them as *G. fragariae* Klebahn, a species hitherto unrecorded from North America (Alexopoulos and Cation, 1952).

In September, 1949, a number of isolations from diseased leaves of strawberries collected from Minnesota gave typical perithecia of *Gnomonia fragariae* on cornmeal-malt agar. The development of the ascigeral stage on culture media facilitated a study of the genetics and variation in the species. However, it was noticed that perithecium formation was erratic and uncertain, especially on synthetic media. In earlier attempts to synthesize a medium adequate for sexual reproduction of the fungus, it was observed that most of the isolates of the fungus could not grow well on some synthetic media. As growth is always preliminary to the initiation of reproduction, a definition of the requirements for growth was considered a prerequisite to the determination of the requirements for reproduction. The writer investigated variation in *Gnomonia fragariae*, and the influence of temperature, hydrogen-ion concentration and nutrition on growth and perithecium formation.

MATERIALS AND METHODS

The fungus isolates used in this study were obtained from diseased leaves of wild strawberry in Minnesota. Single pycnidiospores were isolated from the pycnidial ooze on the moist diseased leaves with a micropanipulator using the method described by Dickinson (1933). The single spores were transferred with the micromanipulator to drops of agar on cover slips, where they were allowed to germinate. The hyphae were allowed to grow for a day or two and then were transferred to agar slants.

Natural and synthetic media were used in these studies. Potato-dextrose agar and cornmeal agar, with or without the addition of malt extract, were usually used. Cornmeal agar was prepared by the addition of 15 gm. of agar to an infusion made from 75 gm. of cornmeal in a litre of water.

For nutritional studies, the basal medium had the following composition:

Dextrose	20 gm.
KH_2PO_4	1.5 gm.
$Mg.SO_4.7H_2O$	.5 gm.
KNO_3	3.12 gm.
Trace elements	—0.5 ml. (Equivalent in p.p.m. to .005 boron, 0.02 copper, 0.10 iron, 0.01 manganese, 0.01 molybdenum, 0.09 zinc.)
Distilled water to make up 1 litre.	
Agar	15 gm.

Vitamins in mixture[2], individual vitamins[3], casein hydrolysate, and other amendments were added according to the requirements of the individual experiment. All of the vitamins were passed through a Zeiss filter, and were added to the autoclaved media.

Except when otherwise stated, all cultural and physiological studies were made at least in triplicate. Twenty ml. of the liquid nutrient solution were used, but 25 ml. of solid media. Except when otherwise stated, all the work was done at room temperature, approximately 25°C.

CULTURAL VARIATION AND DIFFERENTIATION OF THE ISOLATES

On potato-dextrose and cornmeal agar the cultural differences between thirty-eight single pycnidiospore isolates were not clear. Growth of all the isolates was very closely confined to the medium, thus masking most of the mycelial characters.

On cornmeal-malt agar these isolates produced perithecia. Thirty three of them produced perithecia exclusively while the remaining five formed both pycnidia and perithecia. There were decided differences among the isolates both in colony diameter attained by them and in the number of perithecia formed on this medium. (Table 1).

All but two of the isolates came from a single pycnidium. Single pycnidiospore isolates from the same pycnidium differed in colony diameter, amount of perithecium formation, and formation of perithecia exclusively or in association with pycnidia. The colony diameter varied from 46 mm. to 77 mm., the least significant difference being 6 mm. Perithecium formation ranged from poor to excellent: it was poor for 7, moderate for 2, fair for 3, good for 16 and excellent for the remaining 8 isolates under the conditions of the experiment. Four of the isolates formed perithecia in association with pycnidia, while others produced perithecia exclusively. In the course of ordinary sub-culturing, five isolates produced perithecia on potato-dextrose agar, while the others did not.

The addition of malt extract to potato-dextrose agar increased mycelial growth and the cultural characters of the isolates were more prominent on this

2. The stock solution of the vitamin mixture contained 100 mμ moles per ml. of each of thiamin hydrochloride, riboflavin, pyridoxine hydrochloride, p-amino benzoic acid, nicotinic acid and calcium pantothenate, 0.2 microgram per ml. of biotin methyl ester, and 100,000 mμ moles per ml. of i-inositol. Of this stock solution, 0.5 ml. were used per 20 ml. of the medium in each 125 cc Erlenmeyer flask. Ascorbic acid (50 mμ moles per 20 cc. of the medium) provided 0.44 mg. of ascorbic acid per litre of medium.

3. Individual vitamins were used at the same concentration as in the mixture.

TABLE 1. *Colony diameter after six days and perithecium and pycnidium formation after 40 days by single pycnidiospore isolates of Gnomonia fragariae grown on cornmeal-malt agar.*

Isolate	Colony diameter[a] (mm.)	Rating for formation of	
		perithecia[b]	pycnidia[c]
III-3	90	5	..
II-1	69	5	..
35	75	5	..
38	73	5	..
30	72	5	..
33	71	5	..
7	70	5	..
32	67	5	..
39	62	5	..
31	60	5	..
5	77	4	..
10	73	4	..
18	71	4	..
16	69	4	..
29	68	4	..
4	67	4	..
4	67	4	..
36	66	4	..
27	63	4	..
28	60	4	..
37	58	4	..
26	54	4	..
21	53	4	..
19	69	3	..
17	68	3	..
25	63	3	..
13	67	2	..
12	62	1	..
1	60	1	..
23	58	1	..
2	54	1	..
24	53	1	..
3	46	1	..
9	74	4	2
8	70	4	1
6	67	4	2
20	69	2	3
11	64	1	2

a L. S. D.=6 mm.

b 33 isolates produced perithecia exclusively.

c 5 isolates produced both perithecia and pycnidia.

medium than on potato-dextrose, cornmeal or cornmeal-malt agars. All the isolates were then plated on potato-dextrose-malt agar to study other cultural differences among the isolates.

The thirty eight isolates (thirty six of which came from a single pycnidium) could be differentiated into at least six cultural races (Figure 1) on potato-dextrose-malt agar. Three of these races differed in colony colour from white to black. The other three races were differentiated on the basis of the amount of mycelium and the type of growth. They represented respectively, mycelial growth arising as dots, smooth suppressed growth confined very closely to the medium and a thick growth of the mycelium. The number of perithecia produced on this medium was low in general and only ten of the isolates formed perithecia.

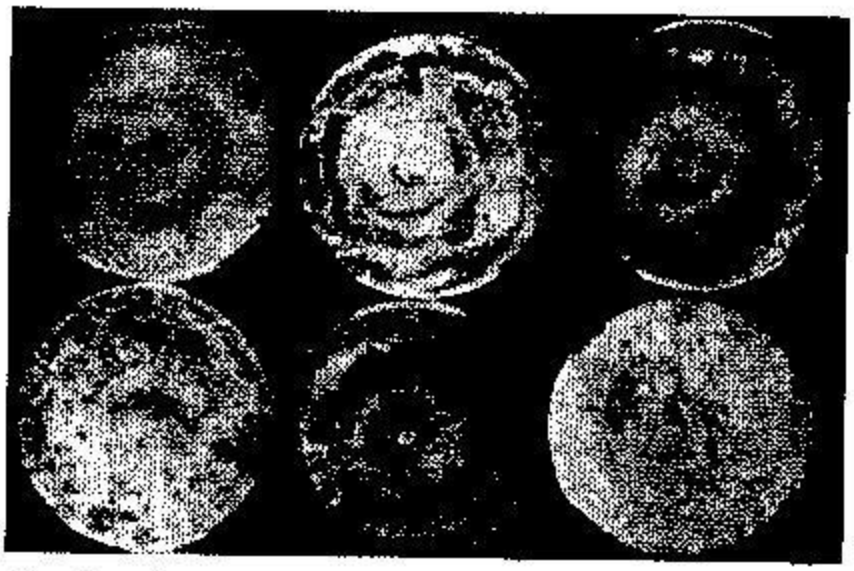

Figure 1. Cultural characters of six races of *Gnomonia fragariae* on potato-dextrose-malt agar.

These results indicate the existence of a large number of biotypes in *Gnomonia fragariae*, which differ in their sporulating ability and cultural characters. The 36 single conidial isolates which originated from a single pycnidium differed in colony diameter and amount of perithecia produced on cornmeal-malt agar, and could be differentiated into six cultural races on potato-dextrose-malt agar.

Four isolates representing different races were selected for further study; temperature and hydrogen-ion concentration relations, and nutritional requirements for growth and perithecium formation. These isolates are designated as races 1, 2, 3 and 4 respectively in subsequent pages. Races 1 and 2 originated from the same pycnidium.

TEMPERATURE RELATIONS

The effect of temperature on growth and sporulation of the races was studied on cornmeal-malt agar (1.0 per cent malt extract). The four races were plated singly, in four replicates, and were incubated at 0°, 5°, 10°, 15°, 20°, 25°, 30°, and 35°C. Growth of all races was sparse and oppressed at all temperatures. Colony diameters were recorded every other day for 8 days, while notes for perithecial formation were taken after 25 days.

The colony diameters attained by these races during 8 days, over the range

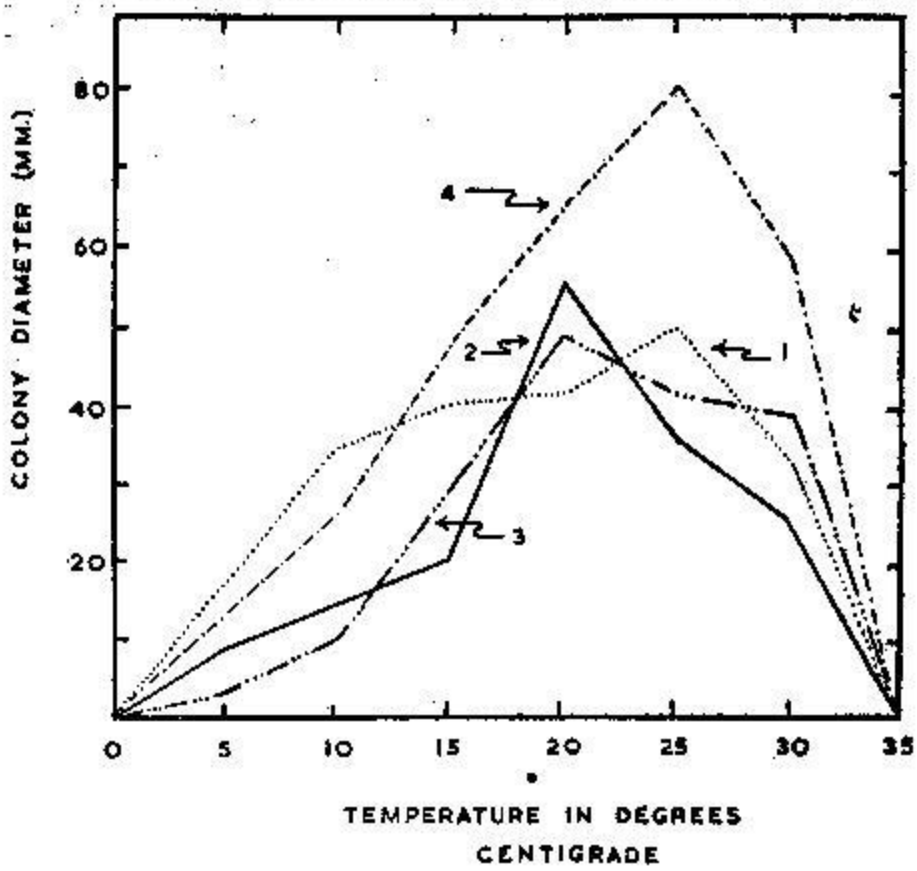

Figure 2. Effect of temperature on colony diameter of four races of *Gnomonia fragariae* after 8 days on cornmeal-malt agar.

of temperatures studied, are summarized graphically in Figure 2. All races grow well over the range of temperatures 5°C. to 30°C. The optimum for two of them was 25°C., while that for the other two was 20°C. Thus, the races fall into two groups as regards optimum temperature for growth.

Races did not develop mature perithecia at 5° to 20°C., inclusive, but perithecial initials were formed in abundance. At 25°C., however, mature perithecia developed after one month. Thus, the optimum temperature for perithecium formation is 25°C on this medium.

RELATIONS OF HYDROGEN-ION CONCENTRATION

The effect of hydrogen-ion concentration on the growth of four races was determined on the basal medium in liquid culture.

The basal medium, containing vitamins and micro elements was adjusted to a pH range of 2.4, 3.2, 3.9, 4.7, 5.5, 6.2, 6.9, and 8.0 by the use of phosphate buffer solutions. The pH ranges of 3.1 to 4.7 and 5.5 to 8.0 were adjusted by the use of H_3PO_4 plus KH_2PO_4 and KH_2PO_4 plus K_2HPO_4, respectively, that of 2.4 and 9.1 were adjusted by the use of H_3PO_4 and K_3PO_4 respectively. In preliminary experiment, the quantities of the buffer solution required to give the desired pHs were determined, and in actual experiment, the buffer solutions and the medium were sterilized separately and later combined. The pH values of these combined solutions were taken to represent the initial pH value in each series. Data on the dry weight of mycelium produced after 17 days are in Figure 3.

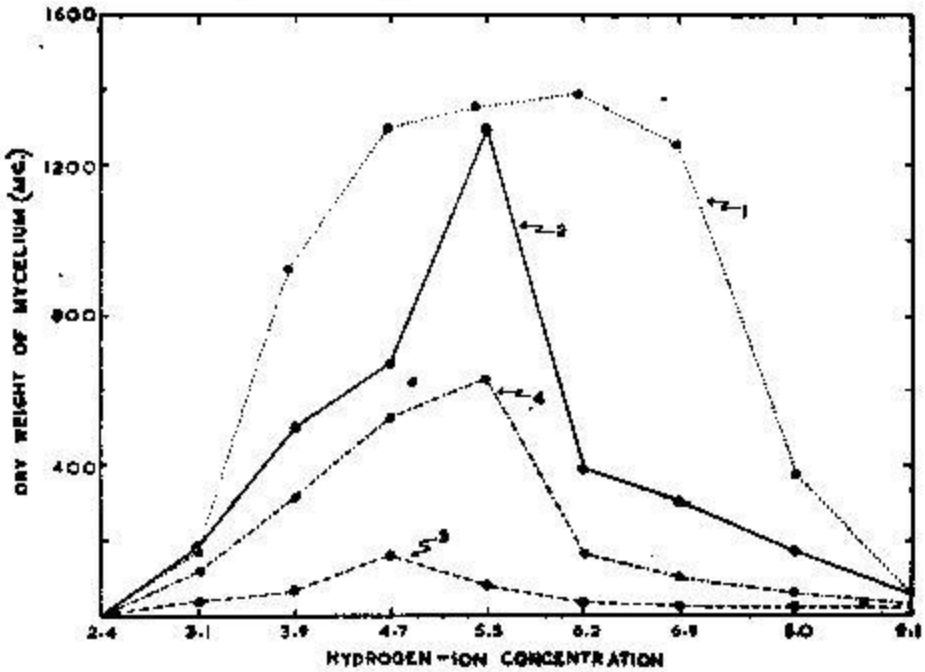

Figure 3. Effect of initial pH of the basal medium plus vitamin mixture on the dry weight of mycellium produced by four races of *Gnomonia fragariae* after 17 days.

None of the isolates grew at pH 2.4 and they grew only a little at pH 9.1. The isolates differed in rate and total amount of growth at almost all the pH values between 3.9 and 9.0. In general, growth was maximum in the range of pH 4.7 to 6.2, the actual maximum being at pH 5.5 for two of the races, at pH 4.7 for race 3 and at pH 6.2 for race 1. However, the curve of growth for race 1 levelled off at pH 4.7 to 6.2.

Sporulation and cultural characters of the two races 1 and 3 were determined on cornmeal-malt agar (malt extract 1.0 per cent) adjusted to different pH values. The two races formed perithecia and no pycnidia over the pH range 3.9 to 8.0. The perithecia did not mature and did not develop beaks in these tests but were most abundant at pH 3.9 and 4.7.

NUTRITIONAL REQUIREMENTS FOR GROWTH

To ascertain the nature of the deficiency of four races for growth, the races were grown on three nutrient solutions, basal solution, basal plus casein hydrolysate (vitamin free), and basal solution plus malt extract respectively. Dry weight of mycelia produced by the races after 20 days, summarized in Table 2, indicated that all of them were deficient for certain growth substances present in malt extract. On the basal solution, growth of all the races was very poor and the addition of malt extract increased growth tremendously. Casein hydrolysate was not beneficial to the growth of four races.

TABLE 2. *The effect of the addition of casein hydrolysate and malt extract to the basal medium on the growth of four races of Gnomonia fragariae after 20 days' growth.*

Medium	Average dry weight [a] (mg.) of mycelium of the races.			
	1	2	3	4
Basal solution ..	73	113	80	157
Basal solution plus Casein Hydrolysate. ..	7	27	5	50
Basal solution plus Malt Extract. ..	1,551	1,050	878	958

[a] Average of 3 replicates.

Similar results were obtained with agar media, when the four races were grown on these nutrient solutions solidified by purified Bacto agar, according to the method used by Robbins and Ma (1945).

Vitamins

The requirements of different races for specific vitamins was determined by the addition of single vitamins to the basal solution at double the rate mentioned under materials and methods. The dry weights of mycelia produced by different races after 20 days' growth (summarized in Table 3) indicated that all races required vitamin supplements for their best growth, but each one had different requirements. In general, the deficiency of most of the races was multiple and the increase of growth by the addition of all the vitamins in mixture was strikingly higher than that by the addition of any of them individually. A summary of the vitamins stimulatory to different races in the order of their magnitude is as follows:

Race 1. p-amino benzoic acid, ascorbic acid, thiamin, pyridoxine, i-inositol, calcium pantothenate and riboflavin.

Race 2. Ascorbic acid, biotin, nicotinic acid, riboflavin and thiamin.

Race 3. Nicotinic acid and pyridoxine.

Race 4. Thiamin, nicotinic acid and pyridoxine.

The effect of certain combinations of vitamins was also studied. Combination of essential vitamins gave an additive effect on the responsive races. Race 2, for instance, was deficient for both thiamin and biotin. It grew almost twice as much on a combination of thiamin and biotin as on either alone. A striking increase in growth by the use of all vitamins in combination is thus explained on the basis of additive effects of certain vitamins.

TABLE 3. *Dry weight of mycelium of four races of Gnomonia fragariae on the basal solution containing nine vitamins singly and in combination after 20 days' growth.*

Vitamins	Average dry weight [a] (mg.) of mycelium of the races.			
	1	2	3	4
None ..	172	107	188	83
Ascorbic acid ..	910	740	717	223
Biotin ..	118	457	90	153
Calcium pantothenate ..	722	322	185	260
Inositol ..	*b*	227	107	127
Nicotinic acid ..	472	400	382	408
P-amino benzoic acid ..	935	119	183	152
Pyridoxine ..	*b*	277	295	362
Riboflavin ..	632	373	202	198
Thiamin ..	883	310	183	443
Nine in combination ..	1,015	952	305	550

a Average of 3 replications

b Mycelial growth was as good as with Thiamin and with ascorbic acid. Perithecia had developed in three replications. Mycelia were not harvested to make further observations on perithecia.

FORMATION OF PERITHECIA

Genetic Studies

As stated by Alexopoulos and Cation (1948), thirty-eight single conidial cultures produced perithecia abundantly on cornmeal-malt agar. The behaviour of the single ascospores in the formation of perithecia, was ascertained by plating seven single ascospores isolated from two asci of a perithecium singly on cornmeal-malt agar. In repeated tests the single ascospore cultures produced perithecia. To ascertain further whether the behaviour of the fungus might resemble that of *Glomeralla cingulata* (Andes, 1941; Edgerton, 1914; Lucas *et al.*, 1944), single-ascospore isolates were plated singly and in different combinations on cornmeal-malt agar. As usual, single ascospore cultures again produced perithecia and those paired in different combinations formed perithecia around their inocula but not along the line of contact of the two mycalia as is the case of heterothallic fungi.

Relation of Nutrients

Perithecia of *Gnomonia fragariae* did not develop on ordinary synthetic media, but they developed freely on cornmeal-malt agar. To determine the vitamin requirements for perithecial formation, the four races were grown on a series of solidified media consisting of basal, basal plus malt extract, basal plus yeast extract, basal plus vitamin mixture, basal plus casein hydrolysate, and basal plus vitamin mixture plus casein hydrolysate. The experiment was duplicated at different times but no perithecia developed. Hwang *et al.* (1947) stated that the flooding of fully grown cultures with sterile water stimulated production of perithecia in homothallic lines of *Hyphomyces solani* and *Gibberella reseum*. This technique was tried without success in the present studies.

Races 1, 2, 3 and 4 were grown on the medium of Lilly and Barnett[4], in which biotin is added to the basal nutrient medium. They were also grown on this medium in which the following were substituted for biotin: 1, thiamin; 2, vitamin mixture; 3, malt extract; 4, yeast extract; 5, vitamin mixture plus casein hydrolysate. There were quadruplicate flasks for each treatment. No perithecia formed in any of the 24 flasks. Neither did Westergaard and Mitchell's medium[5] which contains biotin as a source of vitamins, nor did similar substitutions of biotin in this medium stimulate formation of perithecia.

4. Lilly and Barnett's medium: glucose, 25 gm.; potassium dihydrogen phosphate, 1.0 gm.; magnesium sulphate, 0.5 gm.; fumaric acid, 1.32 gm.; sodium carbonate, 1.2 gm.; casein hydrolysate equivalent to 2.0 gm. casein; biotin, 6.4 μg.; iron, 0.2 mg.; zinc, 0.2 mg.; manganese, 0.1 mg.; purified difcoagar 15 gms.; double distilled water, 1000 ml.

5. Westergard and Mitchell's medium: glucose, 20 gm.; potassium nitrate, 1.0 gm.; potassium dihydrogen phosphate, 1.0 gm.; magnesium sulphate, 0.5 gm.; sodium chloride, 0.1 gm.; calcium chloride, 0.1 gm.; biotin, 5 μg.; trace elements, 1 ml.; purified difco agar, 15 gm.; distilled water, 1000 ml.

These results indicated that the races tested required vitamins other than those tried or in different combinations than those tried.

Of the nine vitamins used in nutritional studies for growth of the four races, ascorbic acid, inositol and pyridoxine stimulated the formation of perithecia by race 1, but not by the others. It has already been shown that these four races are different with respect to vitamin requirements for growth and the fact that only race 1 produced perithecia when ascorbic acid, inositol or pyridoxine were added shows that there also are difference in the vitamin requirements for perithecial formation.

DISCUSSION

Thirty-eight monoconidial isolates of *Gnomonia fragariae* comprised at least six distinct races that differed in one or more of the following characters: cultural characters on artificial media, including diameter, colour, elevation and zonation of colonies; nutritional requirements for growth and sporulation; temperature relations; effect of hydrogen-ion concentration on growth and sporulation; and ability or inability to produce perithecia alone or both pycnidia and perithecia. Six distinct races were isolated from a single pycnidium.

These cultural races can be distinguished far better on certain media than on others. Among the isolates studied, the best medium for development of distinctive cultural characters was potato-dextrose-malt agar. Cornmeal-infusion-malt agar was very much less satisfactory in this respect, but was the best for the development of perithecia. This suggests that it is necessary to find suitable differential media for distinguishing races in culture, just as it is necessary to find suitable differential hosts for determining parasitic races. It is evident also that standardized differential media should be used when it is desired to compare results obtained at different times and in different places.

The four races studied thoroughly for their nutritional requirements needed vitamin supplements for their best growth, but each one had different vitamin requirements. On a synthetic medium devoid of vitamins no perithecia were produced by any of the races but the addition of ascorbic acid, pyridoxine and inositol singly stimulated abundant production of perithecia by one of the races but not by the other three. These races apparently are homothallic, but, even so, they require special nutrients or growth promoting substances to enable them to produce perithecia. It is apparent, also that there are differences in vitamin requirements for perithecia formation, and this might have a decided effect on the production of perithecia in nature and consequent persistence of the organism. These results do show that there is a high degree of genetic diversity within the species with respect to vitamin requirements.

The temperature requirements of four races were studied thoroughly. The optimum for two of them was 25°C., while for the other two it was 20°C. on cornmeal-infusion-malt agar. These results suggest that different races might tend to predominate under different temperature conditions in nature. Whether this proves to be true or not, the fact remains that there are distinct differences in temperature requirements among at least some of the races.

A study was made of the hydrogen-ion requirements for the growth of four races. All of them grew somewhat at a range of 3.1 to 9.1 but the optimum for one of them was 4.7, that for two was 5.5 and that for one was 6.2. It appears possible that the relation to hydrogen-ion concentration might have some effect on the ability of different isolates to rot fruit of different varieties and in different stages of development.

The existence of so many races in *Gnomonia fragariae* could be significant in the ecology and pathogencity of the species. It would be interesting to study the relation between cultural characters, vitamin requirements for growth and reproduction of the races, and their ecology and pathogencity. The relationship between all these factors and the epidemiology and control of the pathogen would be an interesting and important study.

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