SOME PHYSIOLOGICAL INVESTIGATIONS OF THE GRAPE BLACK ROT FUNGUS, *GUIGNARDIA BIDWELLII* (Ell.) VIALA et RAVAZ

By

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Bachelor of Science

New Mexico College of Agriculture and Mechanic Arts

State College, New Mexico

1951

Submitted to the Faculty of the Graduate School of

the Oklahoma Agricultural and Mechanical College

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

1955

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1955

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ACKNOWLEDGMENTS

The author wishes to express his sincere gratitude to Dr. F. Ben Struble for suggesting the problem and for giving valuable suggestions and criticism toward the study as well as the preparation of the thesis. Grateful acknowledgment is also due Dr. Walter W. Hansen and Dr. John Thomas for their assistance in the preparation of the thesis. Indebtedness is also acknowledged to Dr. Oran D. Steffey for assistance in preparing the photographs.

LIST OF ILLUSTRATIONS

Page

Fig. 1. Colonies isolated from a black-rot leaf lesion after 3 days at 29° C. Note white sectors in colonies 14

Fig. 2. Temperature relations of G. bidwellii in bean decoction and Czapek's media after 32 days . . 19

Fig. 3. Effect of pH on the growth of G. bidwellii on bean juice and Czapek's media incubated at 29° C for 30 days 22

Fig. 4. Multiple growth optima as exhibited by G. bidwellii over a 34 day period at 29° C on Czapek's with ammonium tartrate as the nitrogen source 25

Fig. 5. Average linear increase in growth at 48 hour intervals for G. bidwellii on potato-dextrose and Czapek's agar at 29° C for 18 days 28

TABLE OF CONTENTS

	Page
INTRODUCTION	1
REVIEW OF LITERATURE	3
MATERIALS AND METHODS	9
RESULTS	12
Inoculation and Isolation Techniques	12
Temperature Relations	17
Effect of pH on Growth	20
Determination of Optimum Growth Period	23
Utilization of Carbon Sources	29
Utilization of Nitrogen Sources	31
Utilization of Vitamins	33
Miscellaneous Attempts at Inducing Sporulation . .	35
DISCUSSION	41
SUMMARY	44
LITERATURE CITED	46

INTRODUCTION

Black rot (Guignardia bidwellii (Ell.) Viala et Ravaz) is the most widely distributed and serious disease of grapes east of the Rocky Mountains.

The disease attacks all green parts of the vines, including leaf blades, petioles, tendrils, shoots, blossoms, and fruits. Fruit infections are responsible for most of the damage of economic importance.

Varieties of grapes commonly grown in Oklahoma are, for the most part, susceptible to black rot. The only practical control of the disease has been through the use of carefully timed applications of fungicides.

One of the objectives of the grape breeding program in the Oklahoma A & M Department of Horticulture has been to develop a satisfactory table grape with resistance to black rot. That resistance to this disease is available has been demonstrated by Demaree *et al.* (7), Barrett (4), and Munson (22).

In order to test the large numbers of seedlings resulting from the breeding program in Oklahoma, it has been necessary to develop a satisfactory inoculation technique. An adequate and satisfactory source of inoculum has been the principle impeding factor in the development of such a technique. While overwintered grape black rot mummies have been successfully used as a source of inoculum these are not always available in the quantities needed for inoculation work.

The purpose of the present investigation was to find some means of inducing the fungus, _G. bidwellii_, to sporulate in culture so that a continuous and readily available source of inoculum would be at hand for testing large numbers of grape seedlings for their reaction to black rot. The approach to this problem has been primarily one of investigating the various factors, nutrition, hydrogen-ion concentration and temperature, known to influence sporulation in other fungi.

REVIEW OF LITERATURE

Several investigators working with the black rot fungus, G. bidwellii, have experienced difficulty in obtaining cultures which would continue to sporulate and produce infection when used for inoculation. The results of such investigators as Reddick (25), Luttrell (19), and Barrett (4), dealing with the sporulation of the black rot fungus in culture, and inoculations, have been contradictory and for the most part inconclusive.

Reddick (25) in his two years of inoculation work with the black rot fungus used ascospores and pycnospores derived from mummied grapes. Infection was never induced even though thousands of inoculations were made onto berries, leaves, and stems, both indoors and outdoors in humidity chambers.

Viala and Pacottet (35) were able to isolate G. bidwellii from infected berries by aseptically removing fragments of pulp invaded by the mycelium. The medium used was unfermented grape juice sterilized at a low pressure. Sporulation was always abundant providing the cultures were grown on unfermented grape juice before being transferred to a liquid or solid medium containing 5 parts per liter of an organic acid and 20 parts per liter of glucose. Among the inorganic acids, phosphoric induced sporulation while nitric, sulfuric and hydrochloric supported only vegetative growth.

Luttrell (18) was able to grow G. bidwellii f. euvitus Luttrell. and G. bidwellii f. muscadinii Luttrell. on 3 per cent malt agar with the formation of pycnospores in 3 or 4 days, providing the

cultures were derived from naturally infected material. Cultures sporulated on the first transfer, but not on subsequent transfers. In his work with black rot on the muscadine type of grapes, Luttrell (19) was able to induce infection on potted grape seedlings providing a moist chamber was used for 48 hours. In all inoculations the inoculum was prepared by macerating pycnidia from cultures or from host lesions in sterile tapwater.

Luttrell (19) was able to demonstrate further that G. bidwellii exists in three physiologic races: G. bidwellii f. euvitis Luttrell. pathogenic to native bunch grapes and Vitis labrusca Linn., V. bourquina Munson ex Viala and to V. vinifera Linn.; G. bidwellii f. muscadinii Luttrell, pathogenic to muscadine grapes, (V. rotundifolia Michx.), and to V. vinifera; and G. bidwellii f. parthenocissi Luttrell., pathogenic to Virginia creeper, Parthenocissus quinquefolia (L) Planch. and Boston ivy, P. tricuspidata (Sieb & Zucc.) Planch.

Barrett (4) tried various media in an effort to induce sporulation of G. bidwellii and found malt extract plus thiamine, 50 ug per liter, produced heavy sporulation. Without thiamine the fungus would not sporulate on a second transfer. Lima bean agar and bean broth as a liquid medium also gave good sporulation. Inoculations using inoculum from cultures were not successful on any species, selection or clone of grapes; however, inoculations were successful on many species, species combinations, clones and selections of grape if the inoculum was derived from natural sources. A simple and efficient method for inoculating grapes was employed by Barrett (4) and consists primarily in atomizing a spore suspension, derived from infected grape berries or leaf lesions onto the foliage of grape

seedlings susceptible to black rot. After inoculation the grape seedlings were placed in special humidity chambers for 48 hours and then incubated for 14 days.

Barnett *et al.* (3) and Lilly *et al.* (14) in their studies on the sporulation and physiology of *G. bidwellii*, report this fungus as sporulating on a synthetic medium where maltose and ammonium tartrate were used as the carbon and nitrogen sources. Inoculation of favorable agar media, either with mycelium or a few conidia at one point, resulted in abundant sporulation after 10 to 20 days. Where a heavy suspension of conidia was used as a source of inoculum for plates, sporulation occurred in 3 days. In a later paper, Timnick *et al.* (31) retracted this work when the organism which had been used in the physiological investigations was discovered to have been *Melanconium fuligineum* (Scribner & Viala) Cav. instead of *G. bidwellii*.

Reviews on various phases of investigations concerning nutritional studies on the fungi have appeared from time to time but among the more comprehensive have been those by Steinberg (29, 30) and Lilly and Barnett (16), who include the nutritional requirements of fungi from the standpoint of carbon and nitrogen compounds as well as mineral and vitamin requirements for growth and reproduction. Riker and Riker (26) have listed several methods which have been used successfully in inducing different fungi to sporulate in culture. In general the following are factors which have been found to affect sporulation: concentration of medium, carbon and nitrogen sources, carbon/nitrogen ratios, essential micro elements, vitamins, and environmental factors.

Leonian (12) investigated the physiological relations of some

twenty representative members of the Sphaeropsidales and organized them into groups which were typical in reaction from the standpoint of pycnidium production. Among the factors he found influencing sporulation were presence or absence of light and concentration of nutrients. A high nutrient concentration or a sudden increase in nutrient concentration generally favored fruiting. Not only the amount of nutrients but the proper balance between the constituents of the medium was essential for maximum sporulation.

Various nitrogen sources have been reported as inducing fungi to sporulate, for example, Mix (21) states that *Phyllosticta solitaria* E. and E. will sporulate utilizing nitrate nitrogen. Lilly and Barnett (15) list *Phoma betae* Fr., *Septoria nodorum* Berk., and *Choanephora cucurbetarum* (Berk. & Rav.) Thaxt. as favoring various sources of nitrogen for sporulation.

Specificity of carbon sources for sporulation also occurs for some of the fungi and has been indicated by Hawker (9) who reports *Melanospora destruens* Shear as utilizing glucose and fructose for sporulation. Mix (21) states that *Phyllostictia solitaria* utilizes various hexose sugars for the production of pycnidia. Timnick *et al.* (32) state that *Diaporthe phaseolorum* (Cke & Ell.) Sacc. var. *batatatis* (Harter & Field) Wehm. utilizes lactose for the production of perithecia.

Westergaard and Mitchell (38) have indicated in their work with *Neurospora crassa* Shear and Dodge that the ratio of carbon and nitrogen in a medium may also influence the ability of an organism to sporulate in culture.

Essential micro elements have also been reported as influencing

the rate of sporulation by certain fungi. Extensive work on this, using Aspergillus niger van Tiegh. has been reported by Steinberg (30). Perlman (24) has summarized the data on the micro nutrient requirements of the fungi.

Improved sporulation because of added vitamins has frequently been observed. Barnett and Lilly (2, 1) report thiamine as improving sporulation of Ceratostomella fimbriata (Ellis and Halst.) Elliott while biotin affected sporulation of Sordaria fimicola Ces. and De Not. A review of vitamin requirements of fungi has been presented by Robbins and Kavanagh (27).

Of the environmental factors which have been used to induce sporulation, temperature, light, and hydrogen-ion concentration have been the most important. Barnett and Lilly (2) in working with Ceratostomella fimbriata induced perithecia and ascospore formation at 25° C within 11 days, while perithecia failed to form at 18° C for 60 days. Mathur et al. (20) reported that 15 to 20° C favors conidium formation by Colletotrichum lindemuthianum (Sacc. and Magn.) Bri. and Cav. in culture. Henry and Andersen (10) state that a temperature of 28° C was optimum for sporulation of Piricularia oryzae Cav. while lower or higher temperatures of incubation decreased the number of spores produced. Light has often appeared as a factor which helps induce sporulation. Leonian (12) has shown that Sphaerographium fraxini (Pk.) Sacc. produced pycnidia in light at room temperature, while no pycnidia were formed in the dark at room temperature. Some other organisms forming pycnidia at 8° C were: Melanconium betulinum Schm. & Kz., Naemospaera sp., Pestalotia guepinia Desm., Phoma urens E. & E.,

Phyllosticta opuntiae Sacc. & Speg., *Sphaerographium fraxini* and *Sphaeronema pruinosum* B. & C. According to Coons (6) light favored pycnidial formation by *Plenodomus fuscomaculans* (Sacc.) Coons.

The hydrogen-ion concentration of a basal medium, is another important factor to be considered in inducing various fungi to sporulate. Lilly and Barnett (13) state that *Sordaria fimicola* would not form perithecia until the pH of the culture medium was 6.5 or more. Lockwood (17) studied the formation of perithecia and asci by *Penicillium (Carpenteles) javanicum* van Beijma, *Aspergillus herbariorum seor minor* (Mangin) Thom and Church, and *Chaetomium globosum* Kunze in buffered media of various hydrogen-ion concentrations and found that from a pH of 2.1 - 2.5 the perithecia produced asci with few ascospores or none at all.

MATERIALS AND METHODS

The isolate of G. bidwellii used in the present investigation was originally derived from a single pycnospore in 1951. This culture, Ia, had been maintained under sterile mineral oil and was morphologically unchanged from the original. That the culture was still pathogenic was established in the spring of 1954 when spores were produced on a bean decoction medium and used to inoculate susceptible grape seedlings. Unless otherwise specified, this culture is the one used in all the following experiments.

In order to study carbon, nitrogen, and vitamin nutrition of the grape black rot fungus, it was necessary to select a basal synthetic medium on which the organism would grow well. On the basis of preliminary observations a modified Czapek's medium was chosen. The composition of this medium was as follows:

KH_2PO_4 - - - - - - - - - -	1.0 g
$NaNO_3$ - - - - - - - - - -	2.0 g
$MgSO_4$ - - - - - - - - - -	0.5 g
$FeSO_4$ - - - - - - - - - -	0.01 g
KCL - - - - - - - - - -	0.02 g
Glucose - - - - - - - - -	10.0 g
Thiamine hydrochloride -	100 ug
Biotin - - - - - - - - - -	5 ug
Distilled water to make	1000 ml

The salts used in preparing this medium were of analytical reagent grade. Amino acids, vitamins, and certain carbon sources

used in later experiments were obtained from the Nutritional Biochemicals Corporation. Thiamine and biotin were added to the basal medium because, from preliminary observations, they seemed to promote better growth.

The basal medium was prepared in lots of 10 to 20 l and the pH was adjusted to 4.5. In individual tests the proper amounts of carbohydrate, nitrogen, organic acid, or vitamin were added to separate aliquots of the basal medium. This insured that the basal medium for any given test was uniformly the same.

Six-ounce glass prescription bottles with cotton plugs and screw caps were used as culture bottles. Before each use the bottles were treated with potassium dichromate-sulfuric acid cleaning solution and then rinsed four times with distilled water and dried. To each bottle was added 50 ml of medium. Sterilization was carried out in an autoclave at 15 lb steam pressure for 20 minutes, except with the several carbon sources which were sterilized separately at 10 lb for 20 minutes. The bottles were removed from the autoclave as soon as the pressure was down to prevent possible caramelization of sugars.

The fungus for seeding bottles or plates was grown on potato-dextrose agar slants for 5 days at 29° C. Bottles to be seeded were allowed to stand 48 hours following sterilization after which time the fungus mycelium was added to each in pieces approximately 2 mm square.

Behavior of G. bidwellii on the various liquid media was evaluated by observations for possible spore production and by determining dry weight of the mycelium produced. Mycelium was harvested

by filtering onto pre-dried and weighed filter paper, washed with 100 ml of distilled water, and dried in covered petri dishes at 90° C for 48 hours. After drying, the mycelium was kept in a dessicator until weighed. An analytical balance was used for weight determinations.

In certain experiments the fungus was grown on solid media. Here observations were made for sporulation and growth was evaluated by periodic colony diameter measurements.

Other materials and methods used to a limited extent are indicated in the appropriate section.

EXPERIMENTAL RESULTS

A. Inoculation and isolation technique

Viala and Pacottet (36) stated that the black rot fungus had sporulated well on a medium of bean juice plus sugar and organic acid. Such a medium was prepared to test the behavior of the black rot isolate with which we were dealing. The ingredients of the medium used were as follows: decoction from 200 g of dried beans, 20 g sucrose, 2.5 g tartaric acid, and distilled water to make 1,000 ml. This medium had an initial pH of 3.0 after autoclaving. Cultures in this medium were incubated at room temperature, approximately 20-28° C, and observed at about weekly intervals for spores. By the end of 56 days the fungus was sporulating well. Inoculum was prepared from these sporulating cultures by macerating entire cultures in a blender. The resulting suspension of spores and chopped mycelium was atomized onto four black-rot-susceptible grape seedlings in the greenhouse. A clear plastic bag containing a wad of wet cotton was pulled over each inoculated plant and tied firmly to close the bag. The bags were left on the plants for 48 hours. During the course of this experiment the greenhouse temperature was averaging 90° F, daytime.

Fifteen days following inoculation, a single black rot lesion appeared on each of two leaves of one plant. These lesions were removed, pieces of them surface sterilized in sodium hypochlorite solution, and plated onto potato-dextrose agar. After 3 days incubation at 29° C there were abundant spores on the dark colonies which

had grown from the leaf tissue. Transfers of sporulating colonies to potato-dextrose agar and cornmeal agar continued to sporulate abundantly; transfers of sporulating colonies onto oatmeal agar, grape-berry decoction agar, lima bean agar, and prune agar all failed to sporulate. Subsequent transfers to potato-dextrose or cornmeal agar failed to produce spores. In other words, sporulating cultures could not be induced to continue sporulating past the first transfer from the original culture. This same phenomenon was observed by Luttrell (19). Both Reddick (25) and Barrett (4) report isolating the fungus and from Reddick's work it is not clear whether or not sporulation continued past the original culture. Barrett reports that only some of his cultures sporulated on original isolation and none sporulated past the first transfer without the addition of thiamine hydrochloride to a malt extract agar medium.

In the present investigation spores from a first transfer on cornmeal agar were used in suspension to inoculate susceptible grape seedlings. By the end of 15 days there were many lesions evident on leaves of these plants. The fungus isolated from these lesions behaved in a manner similar to that described above with respect to producing spores.

That the isolate of G. bidwellii with which we were working was unstable is indicated by the variation in the several cultures isolated from inoculated plants Fig. 1.

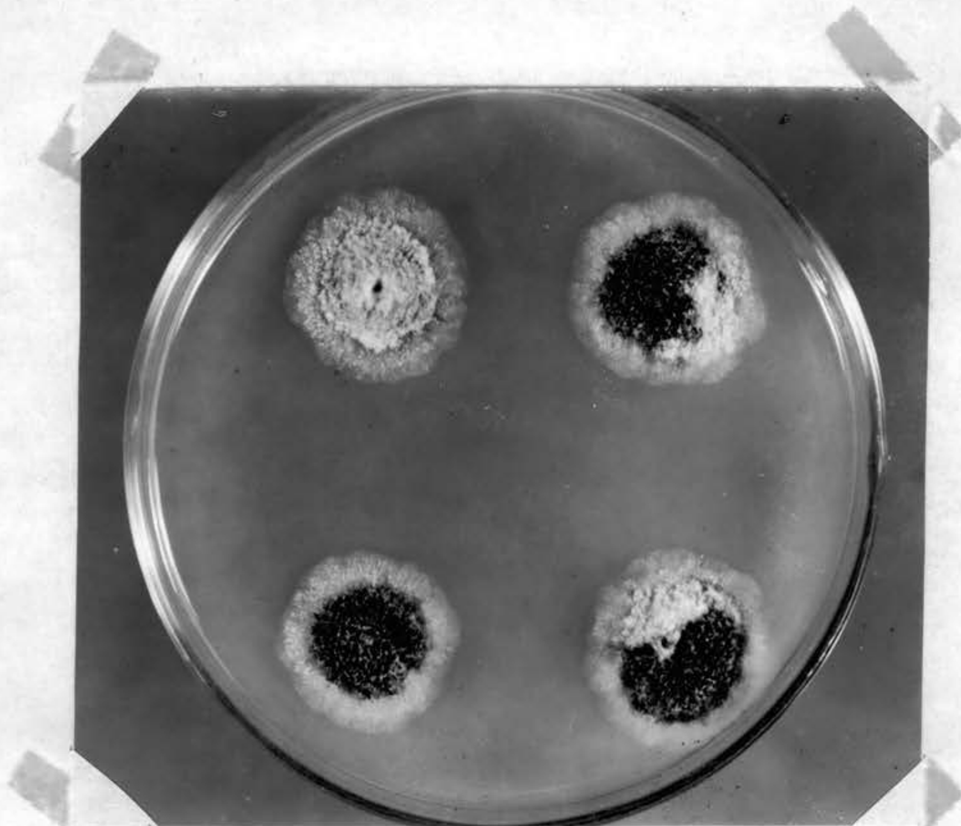

Fig. 1. Colonies isolated from a black-rot leaf lesion after 3 days at 29° C. Note white sectors in colonies.

ATHMORE PARCHMENT
100% RAG U.S.A.

A characteristic colony is black and soon comes to have a surface crust. Many colonies isolated were completely white and many of the black colonies produced white sectors. These white colonies or sectors were never observed to sporulate under any circumstances; as they grew older, they became progressively grayer and finally, in about 10 days, turned black. However, they still did not sporulate. Transfers from colonies or sectors which were originally black were apparently stable. The variations noted were observed only when this organism was first reisolated from diseased tissue or when it was grown on a decoction from green beans. Barrett (4) also notes the black rot fungus as isolated from diseased tissue as being very unstable.

The fact that typical black rot lesions were produced by inoculation on leaves is perhaps worth noting since the success of other workers in this respect has been quite variable. Reddick (25) reports having made several thousand inoculations, presumably some with spores derived from cultures, with all of them yielding negative results. Luttrell (19) mentions no difficulty in infecting susceptible grapes using pycnospores grown in cultures. Repeated attempts by Barrett (4) to infect grape plants with the black rot fungus, using spores from cultures as inoculum, met with failure.

It has not been possible to work further with sporulating cultures of the black rot fungus in the present investigation. Since the early success with the bean decoction medium all attempts at inducing sporulation at other than trace levels have failed.

Several attempts have been made using finely chopped mycelium of _G. bidwellii_ as inoculum but these have never produced a single

lesion on susceptible grape seedlings. Successful inoculations have been made many times using macerated black rot mummies from the previous seasons grape crop. Mummies held several days in moist plastic bags before being used have generally given best results. Even this technique though has not always resulted in infection on grape seedlings.

B. Temperature relations

It is a well established fact that temperature is an important factor in growth and sporulation in the fungi. For G. bidwellii, Viala and Pacottet (37) have reported optimum temperatures for growth and sporulation over a range from 20° to 30° C. They found pycnidia produced over a range from 12° to 35° C. At the lower limits about 1 month was required for the appearance of pycnidia, while at temperatures from 20° upward only 3-10 days were necessary.

Temperature relations of the isolate of G. bidwellii used in the present work were investigated on two different media, Czapek's basal and bean decoction. The media were made as previously outlined and the pH of each adjusted so that after autoclaving the Czapek's had a pH of 4.6 and the bean a pH of 4.5. Seeded replicates were incubated for 15, 30, or 45 days at temperatures of 14°, 18°, 22°, 26°, 29°, and 32° C. Five replicates were harvested at each time period at each temperature and handled as previously described to determine growth as measured by dry weight of mycelium. Observations for sporulation were also made.

Table 1. Effect of temperature on the growth of G. bidwellii after 30 and 45 days on two different media.

Temperature	Dry weight of mycelium after stated number of days:			
	30		45	
	Bean	Czapek's	Bean	Czapek's
°C.	mg	mg	mg	mg
14	70	19	125	22
18	83	26	187	28
22	83	28	193	29
26	145	31	198	31
29	158	37	202	34
32	59	17	86	18

The results of this experiment are presented in Table 1. Data for the 15 day growth period are omitted because growth at this time was so slight that it was considered of no significance. It will be noted from these data that on the Czapek's medium the fungus grew an apparent optimum amount over the range of temperatures used except at the extremes. After 45 days, growth on this medium was essentially the same as that at 30 days. On the bean decoction medium the fungus continued to produce appreciable amounts of mycelium up to 45 days when the experiment was terminated. At the 30 day period on the bean medium the optimum temperature for growth is between 26° and 29° C, while at the 45 day period good growth resulted over the temperature range except at the extremes. Growth data for the subsequent experiments 29° C was used as the temperature for incubating cultures.

At no temperature, on either medium for any of the time periods involved, were spores observed. Cultures on both media incubated at 29° C continued to be negative for spore production up to 75 days.

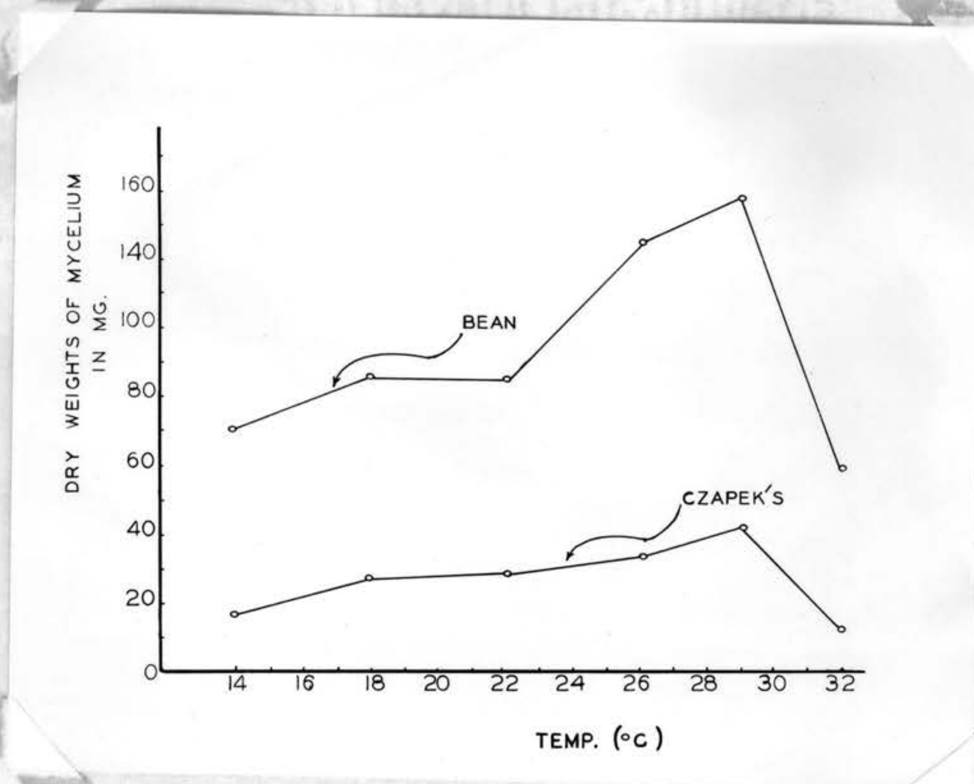

Fig. 2. Temperature relations of G. bidwellii in bean decoction and Czapek's media after 32 days.

C. Effect of pH on growth

Since the work of Viala and Pacottet (37) had indicated that pH might be a factor in growth and sporulation of the black rot fungus, it was considered important that this be investigated. Further, in order to continue with other physiological investigations it was important to determine the pH relations of this fungus.

Two media were used in this experiment, the basal Czapek's and bean decoction made as previously described. Hydrogen-ion concentration was adjusted either with 1 molar sodium hydroxide or tartaric acid over a range from pH 3.0 to 7.0. Initial pH values as recorded in Table 2 were measured following autoclaving of the media. Since the time period for optimum growth of the fungus was not known, replicates on each medium were harvested at each of 4 time periods, 15, 30, 45, and 60 days. Three replicates were harvested at each pH for each of the various time periods. Since there was very close agreement between replications at each measurement only average pH values and dry weights are recorded. All cultures were incubated at 29° C.

Results of this experiment are presented in Table 2. As will be noted from these data there is no single initial pH that produces a distinct optimum of growth. There is evident, however, a range of pH over which this organism grows better than at lower or higher initial pH levels. This range extends from pH 4.5 through pH 5.5. As might be expected the optimum pH level for growth varies slightly, depending on the medium. The optimum pH in Czapek's medium is slightly higher for certain growth periods, viz., 15 and 30 days, than is the

optimum in bean decoction. In general there seems to be a tendency, as indicated by final pH readings, for the pH values of each medium to drift from the extremes of the initial range used toward an average of the range.

Table 2. Effect of pH on the course of growth of G. bidwellii on two different media after each of four time periods with pH of medium for each period.

Initial pH	Average dry weight of mycelium after stated number of days:				Average pH after stated number of days:			
	15 mg	30 mg	45 mg	60 mg	15 pH	30 pH	45 pH	60 pH
Bean decoction medium								
2.9	36	61	28	19	3.0	3.0	3.0	4.0
3.4	39	72	83	69	3.5	3.3	4.5	4.4
4.0	44	78	53	68	4.0	3.5	4.6	5.0
4.5	85	92	96	176	4.5	4.3	4.2	5.2
5.0	61	84	89	194	4.9	4.3	3.8	6.8
5.5	44	79	97	189	5.5	5.3	4.5	6.7
6.1	82	67	115	152	5.9	4.5	4.0	5.1
6.5	71	67	83	76	6.5	5.5	4.5	7.1
6.9	52	51	86	31	6.9	6.3	5.5	7.0
Czapek's Medium								
3.0	4	6	9	1	3.0	3.2	3.1	4.1
3.5	6	8	12	2	3.5	3.2	3.5	3.5
3.8	13	19	57	1	3.8	3.1	3.2	5.9
4.5	19	22	60	135	4.5	3.1	3.2	6.1
5.0	17	35	41	104	5.0	3.1	3.1	3.2
5.5	17	29	39	80	5.5	3.1	3.2	6.3
6.0	17	27	38	87	6.0	3.2	3.2	4.9
6.5	12	18	37	76	6.5	3.6	3.6	3.3
7.1	11	15	32	38	6.9	3.5	3.5	3.2

A growth period of about 30 days was later chosen as the point to terminate most of the experiments measuring growth on a liquid medium. While this time period did not produce maximum yields, as will be noted in Table 2, the amount of growth with the several initial pH values is relatively the same as for the 60 day period

when the highest yields were obtained.

In Fig. 3 the data for the 30 day period are presented to show the effect of the varying initial pH levels on growth.

No sporulation was observed at any pH level regardless of the time period. Observations were made up to 75 days at all pH levels.

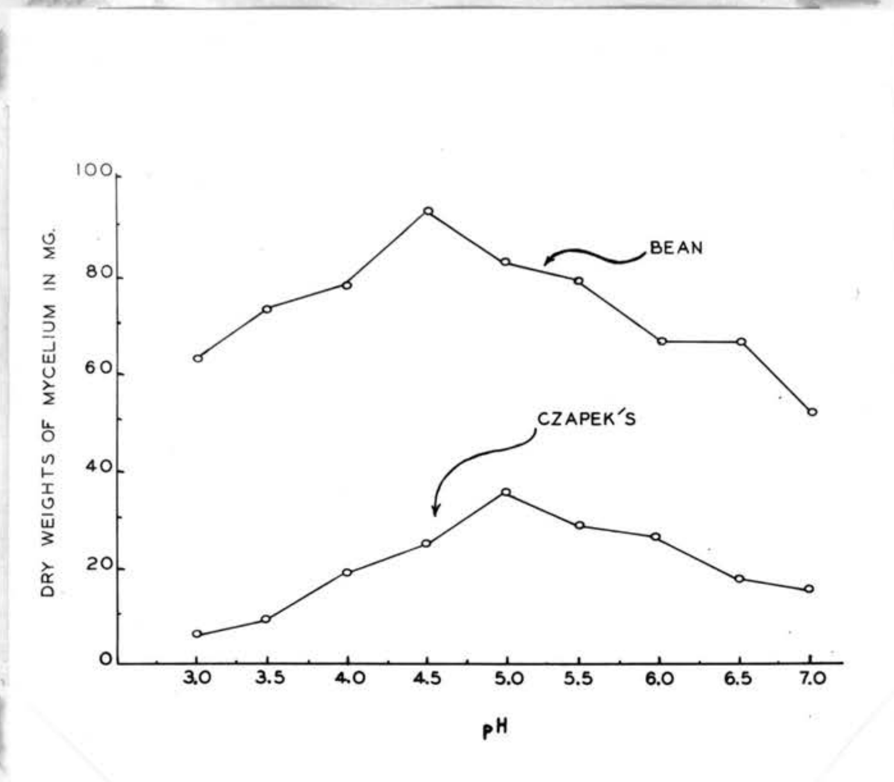

Fig. 3. Effect of pH on the growth of G. bidwellii on bean juice and Czapek's media incubated at 29° C for 30 days.

D. Optimum Growth Determinations

In order to establish the conditions under which it might be expected that G. bidwellii would grow best in culture, it has been necessary, among other things, to determine an optimum growth-time period. That is, after how long an incubation period can growth of this fungus be expected to reach an optimum? Admittedly such a growth-time period established for a given set of conditions might be subject to variation as the several environmental or nutrient factors were varied.

In preliminary investigations on the ability of G. bidwellii to utilize various carbon and nitrogen sources and before the optimum time period for growth had been determined, cultures were harvested at 10 day intervals over a period of 60 days. Results of these studies revealed that there was more growth at the end of 10 days than at the end of 20 and more at the end of 30 days than at 20. At the end of 40 days growth was about the same as it had been for 30 days. In other words, as revealed by these experiments, this fungus seemed to have more than one optimum growth period.

The objective of the experiments to be presented in this section was twofold: (1) to determine insofar as possible an optimum incubation period for evaluating growth, and (2) to determine whether or not there was a single growth-time optimum or several.

The culture medium used was Czapek's made as previously described. This medium was used also with ammonium tartrate, 1.79 g per liter substituted for $NaNO_3$ as the nitrogen source. This substitution was made on the basis of a preliminary evaluation of nitrogen sources in

which ammonium tartrate was found superior to $NaNO_3$. Each of these two media was adjusted so that after autoclaving the pH was 4.6. Five replicates were harvested on each medium at each time interval. Cultures on the Czapek's basal medium were harvested at 5 day intervals from 5 through 35 days while those on the medium with ammonium tartrate were harvested at 48 hour intervals from 12 through 34 days. Dry weight of yields was obtained as previously described. In the one case yields were not taken before the twelth day because growth was not sufficient to measure.

Since the data on the Czapek's with ammonium tartrate are the more detailed and since the results on Czapek's with $NaNO_3$ show essentially the same trends, those data from this latter medium are not presented.

Table 3. The course of growth of _G. bidwellii_ over a 34 day period at 29° C on Czapek's with ammonium tartrate as the nitrogen source.

Incubation time	Av. dry weight of mycelium
days	mg
12	0
14	12
16	8
18	5
20	2
22	9
24	13
26	11
28	20
30	17
32	26
34	22

The data as presented in Table 3 and Fig. 4 show that, under the conditions of this experiment, this fungus, _G. bidwellii_, has

no clear cut single optimum growth time period. Rather, there are a succession of four optima demonstrated within the time limits of this experiment; presumably there might be more than four if the experiment had been continued beyond 34 days. The observation from preliminary experiments that, at least under certain conditions, this organism has several growth-optimum time periods is confirmed. From these data 28 days was arbitrarily chosen as the optimum growth-time period to be used in subsequent work.

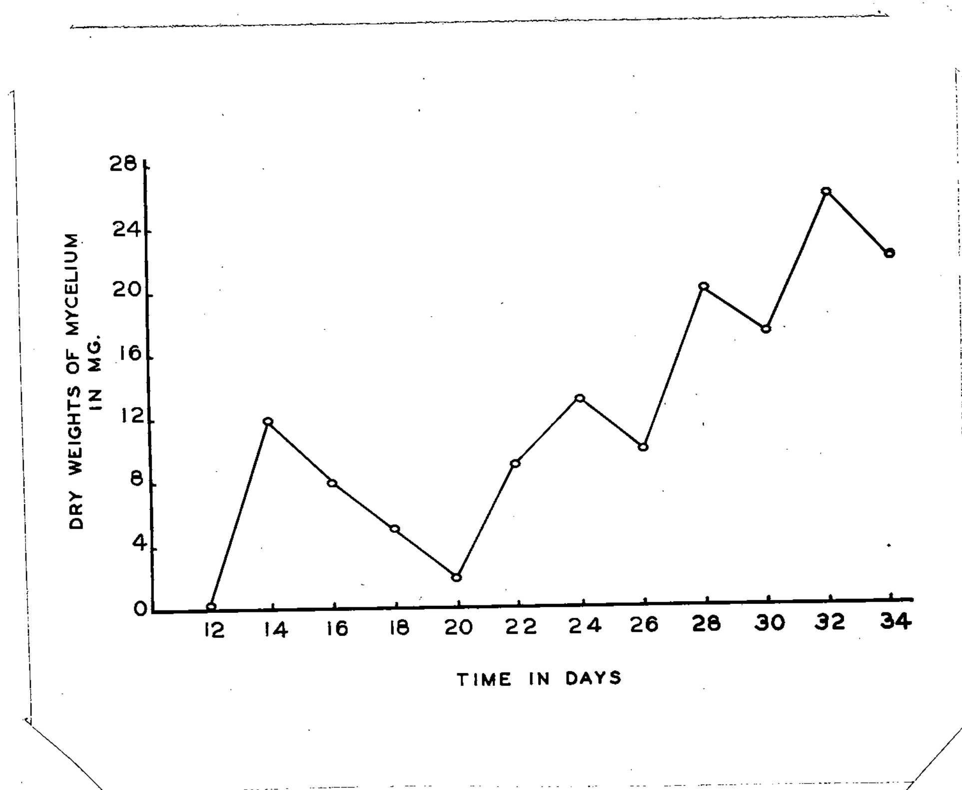

Fig. 4. Multiple growth optima as exhibited by G. bidwellii over a 34 day period at 29° C on Czapek's with ammonium tartrate as the nitrogen source.

In an attempt to obtain further information on the apparent multiple growth optima, growth rate determinations were made on several solid media with G. bidwellii. Potato-dextrose agar and Czapek's agar, with ammonium tartrate substituted for $NaNO_3$, were used in glass growth tubes as described by Ryan et al. (28) to measure the linear growth rate of this fungus. The potato-dextrose agar was adjusted so the pH was 4.8 after autoclaving; pH of the Czapek's agar was 4.5. Two tubes of the potato-dextrose medium and five of the Czapek's medium were incubated at 29° C following seeding. Linear growth was measured in mm at 48 hour intervals as indicated in Table 4.

Table 4. Linear growth of G. bidwellii on potato-dextrose and Czapek's at 29° C over an 18 day period.

Incubation time	Linear growth and average increase in growth on each of stated media:			
	Potato-dextrose		Czapek's	
days	mm av.	mm increase	mm av.	mm increase
2	0.0	0.0	0.0	0.0
4	16.0	16.0	2.0	2.0
6	29.0	13.0	4.0	2.0
8	33.0	4.0	7.0	3.0
10	41.0	8.0	9.0	2.0
12	53.0	12.0	10.0	1.0
14	67.0	14.0	11.0	2.0
16	73.0	6.0	14.0	2.0
18	81.0	8.0	14.0	0.0

The same two media plus a 3% Difco malt extract agar were used in petri dishes. Twenty-five ml amounts of medium were used in each dish. Plates were incubated at 29° C after seeding. On Czapek's agar colony diameter measurements were made for each of 2 diameters at 48 hour intervals and average diameters determined. On the other 2 media average colony diameters were determined at 5 day intervals.

On each medium at each time interval 5 replicates were used.

The data for growth on the solid media are presented in Table 5 and Fig. 5.

Table 5. Radial growth of *G. bidwellii* on Czapek's, malt extract and potato-dextrose agar at 29° C.

Incubation time	Mean colony diameter and increase in diameter on each of stated media					
	Czapek's		Malt		PDA	
days	mm	mm increase	mm	mm increase	mm	mm increase
2	0.0	0.0				
3						
4	0.0	0.5				
5			0.0	0.0	14.6	14.6
6	1.1	0.6				
7						
8	2.9	1.8				
9						
10	11.9	9.0	16.7	16.7	51.9	37.3
11						
12	13.2	1.3				
13						
14	14.0	0.8				
15			23.4	6.7	71.1	19.2
16	14.8	0.8				
17						
18	19.6	4.8				
19						
20	21.5	8.1				
21						
22	22.4	0.9				

It will be noted from these data that the pattern of growth on the solid media has some of the same features as were previously demonstrated on liquid media. While there is not a measurable loss in growth on the solid media, there are very definite periodic fluctuations in growth as measured by rate of increase in colony size.

On none of the media, liquid or solid, or under any of the conditions tried in the entire foregoing section was the fungus,

G. bidwellii, observed to sporulate.

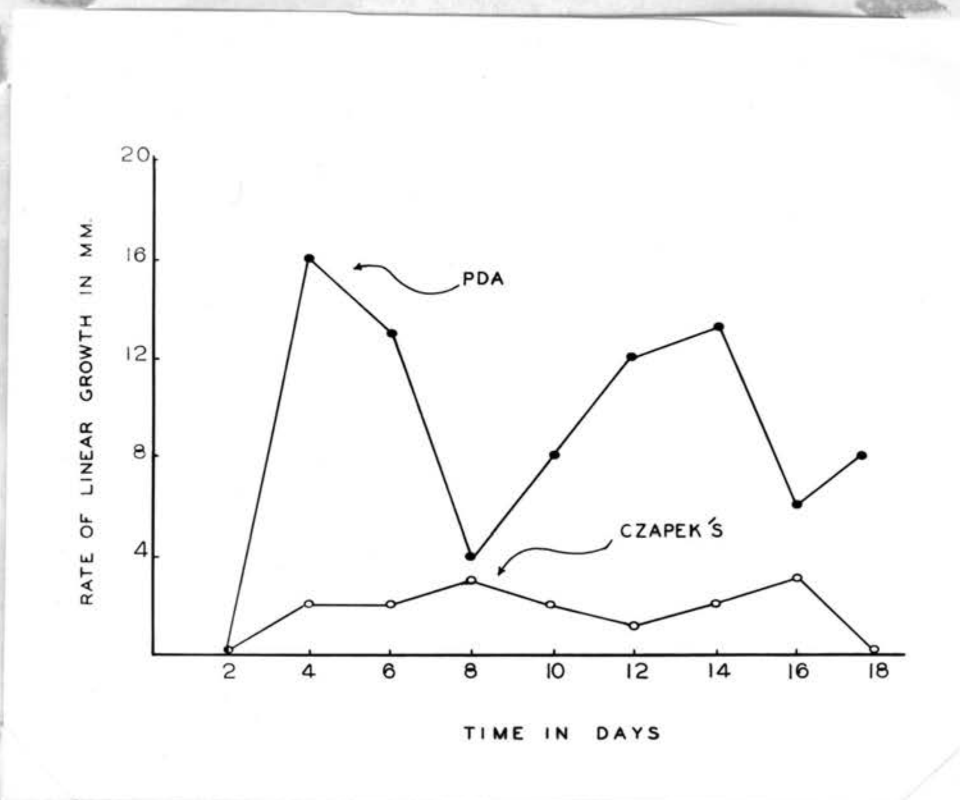

Fig. 5. Average linear increase in growth at 48 hour intervals for G. bidwellii on potato-dextrose and Czapek's agar at 29° C for 18 days.

E. Utilization of Carbon

In previous experiments dealing with temperature, hydrogen-ion concentration, and optimum growth period, the black rot fungus was never observed to sporulate. Since the source of carbon in a medium has been demonstrated (11, 12, 29, 30) to influence sporulation in various fungi, the possibility of inducing G. bidwellii to sporulate by varying the carbon sources was considered.

The Czapek's medium as originally described in section III was used as the basal medium. Carbon sources tested were: monosaccharides-L-arabinose, D-glucose, L-sorbitol, D-xylose, D-galactose, D-mannose, L-sorbose, disaccharides-maltose, lactose, cellobiose, sucrose, trisaccharide-raffinose, polysaccharides-soluble starch and glycogen. All were of analytical grade and were used in amounts calculated to have the same amount of carbon as that contained in 10 g of glucose per liter of medium. Each carbon source was used separately.

Experimental methods and procedures were those already given in section III. Cultures on each carbon source were grown in triplicate at 29° C for 28 days.

The results, using each of these 14 carbon sources individually in the Czapek's basal medium, are presented in Table 4. Mean dry weights of mycelium are presented in each case because the variation in measurements between replicates was of no significance.

The statistical analyses on these data were done by the Statistical Laboratory of the Mathematics Department. An analysis of variance was run on the original data and a least significant difference value

calculated to compare the means. Using the L. S. D. value the means were broken into the 4 groups presented in Table 6.

Table 6. Response of G. bidwellii in Czapek's medium to each of 14 carbon sources as shown by dry weights of mycelium produced. Statistically significant groups of carbon sources are indicated.

Carbon source	Av. dry weight of mycelium	Statistically significant groups at .05 level
	mg	
Cellobiose	19.10	Group I
D-Glucose	18.17	
Maltose	17.33	
Sucrose	16.00	
Raffinose	14.43	
Lactose	14.23	
Glycogen	11.00	Group II
Starch	10.00	
D-Mannose	8.60	
D-Sorbitol	5.83	Group III
D-Xylose	4.90	
L-Arabinose	4.33	
D-Galactose	3.57	
L-Sorbose	.57	Group IV

Tukey's test (33) was used to establish the validity of each group and then a "t" test was used to establish that there was no significance between means within a group.

As can be seen from the data in Table 6, G. bidwellii is not particularly exact in its carbon source requirement. While no one carbon source appears outstanding, the several in Group I, at least, are superior to those in Groups III and IV. Because of the relatively low values obtained here and elsewhere, e. g., a weight of 19.1 mg is the highest, there might be a question as to the biological significance of these data. No spores were observed with any of the several carbon sources in the basal Czapek's medium up to 40 days of incubation.

F. Utilization of Nitrogen Sources

Since manipulations of the environment and nutrients had up to this point given negative results in inducing *G. bidwellii* to sporulate, it was decided to test the possible effect of several nitrogen sources in a medium on growth and possible sporulation. The effects of nitrogen source on growth and reproduction in other fungi have been reviewed earlier in this paper.

The nitrogen sources used in the present work are listed in Table 7.

Table 7. Response of *G. bidwellii* in Czapek's medium to each of 11 different nitrogen sources as shown by dry weights of mycelium produced. Statistically significant groups of nitrogen sources are indicated.

Nitrogen source	Av. dry weight of mycelium	Statistically significant groups at .05 level
	mg	
Asparagin	32.73	Group I
Ammonium Tartrate	27.33	Group II
Yeast Extract	22.53	
Ammonium Sulfate	20.70	Group III
Glycine	20.17	
Malt Extract	16.97	
Casein hydrolysate	19.90	Group IV
Potassium Nitrate	15.07	
Glutamic Acid	15.00	
Sodium Nitrate	10.33	
Czapek's minus nitrogen	9.97	Group V
Urea	5.07	Group VI

The amount of each nitrogen source used was determined by computing the amount of nitrogen in 2 g of asparagin and then calculating for each nitrogen source so that each would provide an equivalent amount of nitrogen. According to Organ *et al.* (23) malt extract contains

approximately 4.5 per cent protein; this was converted to per cent nitrogen and calculated for equivalent nitrogen. The amount of yeast extract used was calculated using the value of 8 per cent nitrogen as reported by Block and Bolling (5). Casein hydrolysate was calculated from the total nitrogen value, 12.7 per cent, given on the original container. Each nitrogen source was used independently in the basal medium, Czapek's, and a control was included with no nitrogen source.

The basal medium and other materials and methods have been previously described in the section on Materials and Methods. All cultures, in triplicate, were incubated for 28 days at 29° C.

While no one nitrogen source was outstanding in the response produced, several appeared satisfactory when compared with those that produced little or no response. Here again since the maximum growth resulting was low there might be a question of the biological significance of the differences obtained.

Sporulation of the black rot fungus was not observed under any of the conditions of this experiment.

The statistical analyses were handled as previously described in the section on Utilization of Carbon Sources.

G. Utilization of Vitamins

According to Robbins and Kavanagh (27) vitamins have been used frequently either to induce or improve sporulation in many fungi. Barrett (4) reported that without thiamine the grape black rot fungus on malt extract agar would not sporulate on a second transfer. In view of this work, a study of the effect of vitamins on the isolate of G. bidwellii with which we were working was undertaken.

The basal medium was Czapek's with no vitamins added and ammonium tartrate, 1.79 g per l, replacing $NaNO_3$ as the nitrogen source. The basal medium was prepared otherwise as noted in Materials and Methods. Each vitamin or vitamin combination was prepared separately and added aseptically to the basal medium. Concentration of the various vitamins used was as follows: riboflavin, 5 ug/l; pyridoxine, 100 ug/l; thiamine, 50 ug/l; inositol, 5 ug/l; biotin, 5 ug/l; nicotinic acid, 250 ug/l. Combinations of vitamins used are presented in Table 8. Other materials and methods were as outlined in the section, Materials and Methods. Each treatment was replicated five times.

Results of this test are summarized in Table 8. Statistical analyses were handled as described in the section on Utilization of Carbon Sources.

Table 8. Response of G. bidwellii in Czapek's medium, containing vitamins or their combinations, as shown by mg of dry mycelium. The three statistically significant groups of vitamins or combinations are indicated in the order of preference for utilization by the black rot fungus.

Vitamin source	Av. dry weight of mycelium	Statistically significant groups at .05 level
	mg	
1. Riboflavin	14.33	Group I
2. Thiamine plus Biotin	12.66	
3. Inositol	9.03	Group II
4. Thiamine	7.43	
5. Pyridoxine plus 2 and 3	6.50	
6. Biotin	5.37	Group III
7. Nicotinic Acid	5.33	
8. All vitamins	5.00	
9. No vitamins	4.40	

From the data presented it appears that G. bidwellii is partially deficient for riboflavin and the combination thiamine plus biotin. Neither thiamine nor biotin alone was sufficient to stimulate growth to the extent that the combination did. With the thiamine-biotin plus inositol and pyridoxine, growth is depressed. Which of the two, inositol or pyridoxine, is responsible for the depressing effect can not be determined from the present data. Evidently one or more of the vitamins is contributing a depressing effect where all vitamins were used together as growth here is no better than with no vitamins.

No one vitamin or combination of vitamins used under the conditions of this experiment induced the black rot fungus to sporulate.

H. Miscellaneous attempts at inducing sporulation

In addition to all of the previous experiments dealing with temperature, hydrogen-ion concentration, optimum growth period determination, carbon, nitrogen, and vitamin requirements of *G. bidwellii*, various other experiments were conducted in an attempt to induce the black rot fungus to sporulate.

The various agar media used in the subsequent tests were all made according to Riker and Riker (26) unless otherwise stated.

A grape juice medium was prepared by using the decoction from 200 g of half-grown bunch grapes. The six-ounce prescription bottles with 50 ml of medium were inoculated and incubated at 29° C for 60 days. A few pycnospores which were produced on this medium were used to inoculate two susceptible grape seedlings with no indication of infection developing.

The possibility of inducing sporulation through the use of natural grape extract which had not been subjected to heat was also investigated. Juice from half-grown bunch grapes was extracted by chopping 100 g of grapes in a Kenmore blender with 50 ml of distilled water and then straining the mixture through four layers of cheese cloth. The grape juice was then sterilized by means of a Seitz filter and 10 ml lots added aseptically to 20 ml of each potato-dextrose agar and water agar before solidification had occurred. Ten ml quantities of sterile grape juice were also placed on top of 20 ml each of potato-dextrose agar and water agar already solidified. No sporulation occurred after 15 days of growth at 29° C on either

potato-dextrose agar or water agar. Vegetative growth was evaluated by using average colony diameter measurements on five plates of each medium. On potato-dextrose agar plus grape extract average colony diameter was 61.5 mm and on water agar plus grape extract colony diameter was 2 mm.

Cultures of the black rot fungus were also grown on the above juice of half-grown grapes for 10 days and then transferred to potato-dextrose agar, lima bean agar, prune agar, and cornmeal agar. Incubation period was for 45 days on these latter at 29° C with no sporulation occurring.

One fact concerning sectoring of G. bidwellii on potato-dextrose agar containing grape juice sterilized through a Seitz filter is worth noting here. The culture, Ia, sectored freely on potato-dextrose agar plus sterile juice just as it had sectored previously upon being reisolated from infected grape leaves as mentioned in the section on Inoculation and Isolation. Although sectoring was abundant with the addition of grape extract to the potato-dextrose agar, no sporulation was ever observed. Twenty-five transfers were made from several black and white sectors with all transfers becoming a normal grayish color within 10-12 days like the original isolate Ia.

Since Viala and Pacottet (35) stated that bunch grape leaves were very susceptible to black rot the possibility of inducing the fungus to sporulate on a medium containing leaves obtained from susceptible varieties of grapes was investigated. Hansen and Synder (8) reported propylene oxide as being used very effectively in sterilizing plant materials and still preserving it close to its natural state; therefore, grape leaves 1-1½ inch in diameter were sterilized

with propylene oxide for 24 hours and then plated onto potato-dextrose agar just before solidification of the medium occurred. The plates were then inoculated as in previous tests with 2 mm pieces of 5-day-old mycelium and incubated for 15 days at 29° C. The surface of the leaves had been covered with approximately 1 mm of agar which received the inoculum. In 15 days the mycelium covered the leaf with a colony 51.5 mm in diameter, with no sporulation occurring.

Maceration of mycelium was also used in an attempt to induce sporulation. Ten-day-old cultures grown on potato-dextrose agar were macerated with a sterile razor blade and then incubated for 45 days at 29° C with no sporulation occurring.

Since the quantity of nutrients sometimes influences the sporulation of a fungus, it was decided to try to induce sporulation by diluting media. The three media, modified Czapek's solidified with 2 per cent agar, bean juice plus 2 per cent agar, and potato-dextrose agar were all diluted with distilled water to 1/2, 1/4, and 1/16 strength. Ten plates for each of the three media were inoculated at each dilution with 2 mm squares of 5-day-old mycelium and then incubated for 15 days at 29° C. No sporulation occurred at any dilution. Vegetative growth was evaluated by comparing the average colony diameters of the 10 plates for each medium at the different dilutions. The average growth in mm for the media at 1/2, 1/4, and 1/16 dilution respectively was Czapek's, 2.0, 0.0, 0.0; bean agar, 9.8, 3.2, 0.0; and potato-dextrose agar, 53.1, 9.2, and 0.0.

Viala and Pacottet (34) state that bean juice with the following organic acids, at less than 5 g per liter, induced sporulation in this order: malic, tartaric, citric, lactic and oxalic. These

five different organic acids at various concentrations were used in an attempt to induce sporulation. The basal medium was decoction from 200 g of beans plus 20 g of sucrose per liter. The organic acid series contained concentrations of from 1 to 5 g of acid per liter of medium. Six-ounce prescription bottles were filled with 25 ml of bean media and then sterilized at 10 pounds steam pressure for 30 minutes. To prevent injury to the inoculum, the bean juice was cooled for 24 hours and then inoculated as in previous experiments. Incubation was for 30 days at 29° C. At one gram per liter concentration the fungus utilized, as evaluated by dry weight measurements of mycelium, the following organic acids in order of preference: citric, tartaric, lactic, oxalic, and malic. The fungus did not grow on lactic, oxalic or malic at a 2 gram per liter concentration or above. In a 3 - 5 gram per liter concentration of organic acid, tartaric was superior to citric as measured by mycelium produced. No sporulation occurred at any concentration of the several organic acids up to 45 days of incubation. Various media such as potato-dextrose agar, raisin agar, prune agar, cornmeal agar, modified Czapek's agar, lima bean agar, nutrient dextrose agar, green bean agar, oatmeal agar, and 3 per cent malt agar were all used in an attempt to induce sporulation. All of the media except the modified Czapek's and green bean agar were made according to Riker and Riker (26). The composition for the modified Czapek's medium was indicated under section III, Experimental Materials and Methods. The green bean agar consisted of the decoction from a No. 303 can of Del Monte brand, Blue Lake, cut green beans, in one liter of water. The modified Czapek's and bean decoction were both solidified with 2.5 per cent agar.

Behavior of the black rot fungus on these various solid media was evaluated by colony diameter measurements. The average diameter measurements in mm for 10 replications on each medium after 15 days growth at 29° C are as follows: potato-dextrose agar, 78.0; raisin agar, 58.0; prune agar, 58.0; cornmeal, 51.0; modified Czapek's, 42.0; lima bean agar, 30.0; nutrient dextrose agar, 29.0; green bean agar, 28.0; oatmeal agar, 28.0; 3% malt agar, 22.5. Indications are that potato-dextrose agar is quite adequate for the vegetative growth of the black rot fungus, while green bean agar supports very little vegetative growth. When the cultures were incubated for 30 days at 29° C sporulation occurred at trace levels only on green bean agar. Spores were not found on any of the other media.

Since sporulation had occurred at a low level on the canned green bean agar, the following experiment was set up to determine the influence of additional carbon, in the form of sucrose, on sporulation. Sucrose was added to the green bean decoction at the following rates: none, 5 gram, and 10 gram per liter. Agar was added as previously described. Each petri dish contained 25 ml of medium and was incubated for 12 days at 29° C. Growth was again evaluated by colony diameter measurements. At the end of 12 days average colony diameters for 10 replications were with no sucrose, 28.9 mm; 5 g sucrose, 67.1 mm; and 10 g sucrose, 75.7 mm. With any amount of sucrose added mycelial growth was stimulated. Sporulation, at trace levels, occurred only where no sucrose was added and after 32 days.

On green bean agar sectoring, as observed previously on reisolation from leaves on potato-dextrose agar and in colonies on Seitz filter sterilized grape juice, was again frequent. With sucrose added to the green bean agar, the frequency of sectoring increased as the amount of sucrose was increased.

DISCUSSION

The conditions necessary for spore production have been established for many fungi as indicated in the literature review. It has been pointed out that both nutritional and environmental factors may influence sporulation. The fungus must also be genetically capable of producing spores. No amount of manipulation of the above mentioned factors is likely to result in spore production by an organism inherently unable to form spores.

That the isolate of _G. bidwellii_ with which we were working was capable of producing spores after approximately $2\frac{1}{2}$ years in storage was established early in these investigations. It was also demonstrated that these spores were capable of inducing disease when inoculated to susceptible grape leaves. While under certain conditions, viz., on green bean agar and on reisolation from diseased leaves, the isolate used, as evidenced by sectoring, appeared unstable, in general there was evidence that it had not mutated.

Under the conditions of the present work, manipulations of medium, temperature, pH, carbon sources, nitrogen sources and vitamin sources have all failed to induce consistent sporulation in the black rot fungus. There has not been opportunity to try combinations of the above mentioned items to any great extent. The factor or combination of factors essential for reproduction in _G. bidwellii_ remains to be discovered.

It is of interest to point out that of those media, on which this fungus did produce some spores, all were derived at least in part from natural plant products. It will be recalled that spores were

found in some instances on the following media: dried bean decoction, heat sterilized grape juice, and green bean agar. Spores on the two latter media were never found in more than trace quantities, and spores were found in quantity only one time on the bean decoction medium. This fungus also sporulated abundantly on potato-dextrose and cornmeal agar, but only through the first transfer, on reisolation from lesions on grape leaves. A possible explanation for this latter phenomenon might be that passage through the host provides a stimulus to sporulation which is lost after the first transfer. The problem might be, then, to in some way provide this stimulus in culture or to maintain it in cultures reisolated from lesions.

Since it has been demonstrated in the present work that this organism is not particularly sensitive to temperature, pH, and carbon, nitrogen or vitamin sources, it might be that the most fruitful future investigations would lie in the area of manipulating nutrient levels and light. Steinberg (30) suggests that too little attention is given the effects of high solar light intensities on reproduction. As he points out many fungi sporulate in nature under conditions of relatively high light intensity. Light may have been an important factor in the failure here to induce sporulation on dried bean decoctions after the early success with this medium. When spores were produced on this medium, incubation had been at room temperature near a window. Subsequent trials with this medium were in dark incubators or in diffuse light at excessively high room temperatures. Shifts from high to low levels of nutrition or vice versa might be indicated by the behavior of the black rot fungus when isolated from leaf lesions.

Evidence as obtained in this investigation indicates that this

fungus grew on certain media in a rather erratic fashion. The fact that there were several apparent growth optima each followed by a real decrease in mycelium is of considerable interest since this phenomenon is not known to occur with other fungi. The data at hand are insufficient to explain this behavior. A possible tentative explanation is that, after each peak of growth, autolysis releases from the old mycelial cells some essential metabolite which is thus again made available for renewed growth by the fungus. This process could conceivably be repeated several times on prolonged incubation. Further attempts at explaining the phenomenon seem unwarranted without further experimentation.

While the present work has not fulfilled the original objective of providing a ready source of inoculum for testing the reaction of quantities of grape seedlings to black rot, there has resulted a suggestion of a method that could simplify the testing process. It will be recalled that reisolations from black rot leaf lesions produced abundant spores on either potato-dextrose or cornmeal agar. With a relatively few good, overwintered black rot mummies as an original source of inoculum, lesions could be obtained on susceptible seedlings in the greenhouse. Reisolation from these lesions should provide inoculum in nearly any quantities desired if the process were timed properly.

SUMMARY

Several aspects of the physiology of the grape black rot fungus, G. bidwellii, have been investigated with the objective of inducing this organism to sporulate in culture so that quantities of inoculum would be readily available for testing the reaction of grape seedlings to black rot.

Among the aspects investigated were temperature relations, effects of pH, time in relation to optimum growth, utilization of a variety of carbon, nitrogen and vitamin sources, and behavior on several natural and semi-synthetic media. A modified Czapek's medium was used as the basal medium for most of the studies.

Since spores were not produced under most circumstances all evaluations were based on the dry weight of mycelium produced on liquid media or on colony diameters where a solid medium was involved. While this fungus grew well over a range of temperatures, 29° C was apparently optimum. A range of pH, 4.5 - 6.0 was satisfactory. Of 14 carbon sources tested, cellobiose, D-glucose, maltose, sucrose, raffinose, and lactose were superior to the others. Of the 11 nitrogen sources asparagin and ammonium tartrate were best with yeast extract, ammonium sulfate, and glycine producing good growth. Sodium nitrate and urea were apparently unsatisfactory as nitrogen sources. The results indicate a partial deficiency in this fungus for the vitamins riboflavin and the combination thiamine plus biotin. Biotin or thiamine alone, nicotinic acid, and pyridoxine plus thiamine, biotin and inositol produced no response.

A variety of natural media including Seitz-filter sterilized grape juice, heated sterilized grape juice, dried bean decoction, green bean agar, grape leaves, oatmeal agar, cornmeal agar, prune agar, malt extract agar, and lima bean agar were all tried in an effort to induce sporulation. Of these, spores were found once on the dried bean decoction medium and several times in trace quantities on the heat sterilized grape juice and green bean agar. On reisolation from leaf lesions produced by spores from the bean decoction, sporulation was abundant on potato-dextrose and cornmeal agar through the first transfer. On none of the other media nor under any of the other conditions in this entire series of experiments were spores observed.

As observed on several media the black rot fungus has no clear cut single optimum growth time period. Rather a succession of optima are indicated. Evidence at hand is insufficient to explain this behavior.

A suggested technique for obtaining quantities of spores for inoculum is outlined. This involves reisolations from leaf lesions produced by spores from a few mummies.

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Thesis: SOME PHYSIOLOGICAL INVESTIGATIONS OF THE GRAPE BLACK ROT FUNGUS, <u>GUIGNARDIA BIDWELLII</u> (ELL.) VIALA et RAVAZ.

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