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A Study on the Growth and Sporulation of Phytophthora megasperma var. sojae

H. H. Ho

Department of Biology, State University College New Paltz, New York 12561, U.S.A.

Phytophthora megasperma var. *sojae* is the fungus causing a disease of soybean commonly known as root and stem rot. The growth of the fungus on agar plates has been briefly studied by some workers, e. g., Suhovecky (1955), Skotland (1955), Hilty and Schmitthenner (1962), using a few common media while the nutritional requirements for carbon and nitrogen were investigated by Herr (1957). Previous workers found that sporangia never developed on solid media, but could be induced to form by various methods, e. g., (a) placing mycelial wefts in Petri's solution (Skotland, 1955); (b) placing washed, diseased tissues in semisolid Difco Lima bean agar (Klein, 1959); (c) treating corn meal culture squares with Petri's solution and then with distilled water (Hildebrand, 1959). In this study, an attempt was made to study some factors which influence the growth and sporulation of *P. megasperma* var. *sojae*.

Nature of Medium

Twenty common media were tested. While many of them were products of Difco Laboratories Inc., Detroit, Michigan, and were prepared as instructed, the others were made up as follows:

Dwarf bean agar — 30 g in 1,000 ml distilled water, 20 g agar.

Malt yeast agar — Difco malt broth, 0.2 per cent yeast powder, 1.8 per cent agar.

Onion agar — Peeled onion 100 g, distilled water 500 ml. Mixture autoclaved for half hour. Decant. Add 10 g agar.

Pea agar — Dried pea powder 200 g, distilled water 500 ml. Mixture autoclaved for half hour. Decant. Add 10 g agar.

Potato agar — Distilled water 500 ml, peeled and sliced potato 100 g. Mixture autoclaved for half hour. Add 10 g agar.

Soil extract agar — Extract prepared according to Barr's method (1965). 1.8 per cent agar.

Various V-8 juice agars — Miller (1955).

Medium (15 ml) was poured into 9-cm petri dishes. Discs cut from the margin of colonies on V8/$CaCO_3$ medium (Miller, 1955) with a

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No. 2 cork borer were used as inoculum. Three replicates were used per treatment and the dishes were incubated at 25 C. At the end of 6 days, colony diameters were measured along two lines drawn at right angles through the centre of each colony (see Table 1).

The whole fungal colony was then cut out with a sterile scalpel to include as much as possible of the advancing hyphal margin. This large disc was submerged in a 9-cm deep petri dish containing non-sterile stream water which was collected and allowed to stand overnight. After

Table I

Growth and Sporangia Formation of P. megasperma var. *sojae* on various Agar Media

Agar media	Mean colony diameter in mm (N = 5—6)	Sporangial production
Bean pod	43.9 ± 5.9	++
Corn meal	52.2 ± 9.0	+
Corn yeast	63.7 ± 6.2	++
Czapek yeast	28.0 ± 5.0	+
Dwarf bean	64.1 ± 4.6	+++
Lima bean	57.3 ± 1.6	+++
Malt	19.4 ± 2.4	+
Malt yeast	17.7 ± 1.9	++
Oat	75.7 ± 1.8	++
Onion	36.0 ± 1.6	++++
Pea	58.8 ± 1.5	+++
Potato	44.8 ± 3.8	+++
Potato dextrose	16.9 ± 1.2	++
Prune	38.8 ± 2.9	+
Soil extract	24.1 ± 7.6	+
V-8 juice	25.6 ± 0.6	++++
V8/$CaCO_3$	75.2 ± 1.8	+++
V8/$CaCO_3$ filtrate	53.0 ± 2.0	++
V8/NaOH	38.1 ± 3.5	+++
Yeast dextrose	34.5 ± 3.7	+
Yeast starch	53.0 ± 0.6	++

incubation at 25° C. for 14 hrs., the water was replaced by fresh non-sterile stream water, followed by another change 1 hour later. Nine hours after the first change of water, fungal activity was stopped by adding several drops of 0.2 per cent mercuric chloride solution with 0.1 per cent Tween wetting agent. Sporangia formed from hyphae on the edges of the colonies were counted under 10 × 10 magnification. Counting was facilitated with the help of a Whipple gridded eyepiece. Three random fields were chosen per colony. The average numbers of sporangia per microscope field were grouped into several broad categories. If the number of sporangia per microscope field was grater than 50, it was designated by ++++; 30—50 by +++; 10—30 by ++; and below 10 by +. Results in Table I

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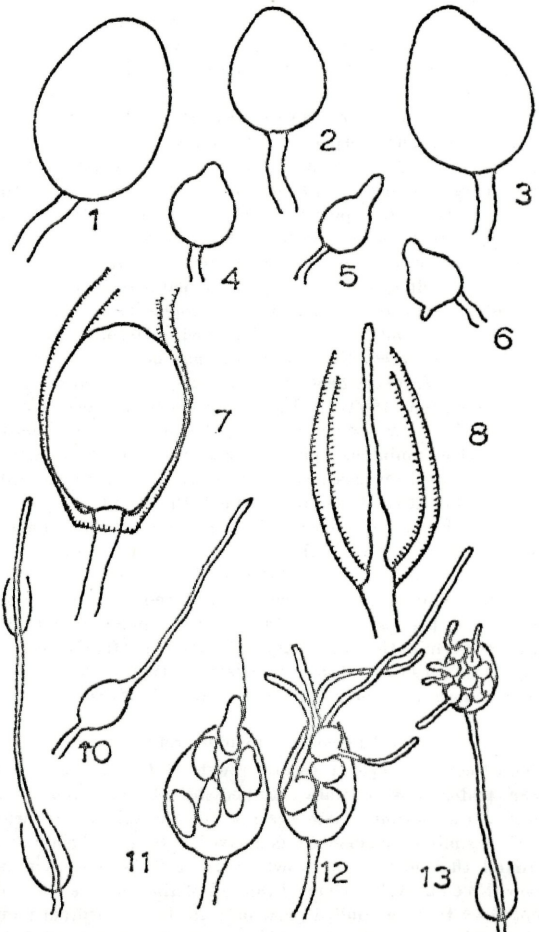

Figs. 1—3. Typical sporangia (× 640). — Figs. 4—6. Papillate sporangia (× 200). — Figs. 7—9. Proliferation of sporangia (Figs. 7, 8, × 640; Fig. 9, × 200); Fig. 7. A third sporangia developing from the base of an empty sporangium, which had proliferated once already. Fig. 8, 9. Hypha growing through base of empty, proliferated sporangium. — Fig. 10. Sporangial germination by germ tube. × 200. — Fig. 11. Zoospore escaping from sporangium. × 640. — Figs. 12, 13. Frustrated dehiscence of sporangia. — Fig. 12. × 400; Fig. 13 × 200.

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represent the average of two separate experiments each with three replicates. Of all the media tested, those which supported fast colony growth and good sporulation, were V8/$CaCO_3$, lima bean, dwarf bean, and pea agars.

The sizes of sporangia were extremely variable, depending on the nature of media. The morphology of sporangia was studied in greater detail, using 20% V8/$CaCO_3$ as the culture medium. The sizes of sporangia even on this one medium varied considerably, as shown in figs. 1—13. The smallest sporangia approached the sizes of "miniature sporangia" described by Drechsler (1931) and gave rise to one or two zoospores only. Sporangia were typically non-papillate (figs. 1, 2, 3) though sometimes appearing slightly papillate to papillate (figs. 4, 5, 6). The term "papilla" was used to denote the nipple-like apex of sporangium (Waterhouse and Blackwell, 1954). Definite proliferation of sporangia was observed. New sporangia may be borne on a new sporangiophore arising from the base within the empty sporangium as described by Hildebrand (1959) or directly on the base (figs. 7, 8, 9). In the former case, the new sporangium may be of the same size as the old one or the size diminishes progressively with the freqrency of proliferation. In the latter case, the sporangia are either slightly or considerably smaller than those first formed. Proliferation may occur more than once within an empty sporangium. Previous workers have described sporangium germination both directly, by formation of germ tube (fig. 10), or indirectly, by production of zoospores (fig. 11). Present observations on sporangium germination on the above medium indicated that the latter process was by far the most predominant. Sometimes, sporangium germination was either abortive or "frustrated" — a term used by Drechsler (1931) to describe the retention of zoospores within sporangia and their germination in situ (figs. 12, 13).

Effect of temperature

The effect of temperature on growth of *P. megasperma* var. *sojae* on agar plates was initially studied by Skotland (1955), who determined the optimum temperature for growth on oatmeal agar to be 24° C. A similar conclusion was arrived at by Hildebrand (1959), who found that maximum growth was at 25° C on corn meal agar. However, Klein (1959) claimed that all of the seven isolates of soybean *Phytophthora* that he studied grew best at 20° C. Cardinal temperatures have been shown previously to be useful for species delimination in *Phytophthora* (Leonian, 1934). Being aware of the various drawbacks in measuring fungal growth by the current method (Hawker, 1950; Cochrane, 1958), the effect of temperatures was studied both by measuring diameters of fungal colony on agar plates and by determining mycelial dry weight in solution culture. For direct comparison, 20% V8/$CaCO_3$ filtrate was used in both cases.

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Effect of temperature on colony diameter

The V8/$CaCO_3$ filtrate was incorporated with 1.8% Bactoagar. Such medium (50 ml) was poured into 140-mm diameter petri dishes, three replicates being used for each treatment. Inoculum was taken from edge of actively growing colonies on V8/$CaCO_3$ agar plates using a No. 2 cork borer. Plates were incubated at 5°, 10°, 15°, 20°, 25°, 30°, and 35° C. The results are recorded in Table II. When the mean colony diameters at the end of 12 days are compared, statistical analysis proved significant differences in growth between consecutive temperatures (all at 0.01 level but 0.05 level between 25° and 30° C.). The optimum temperature for linear growth on agar was thus 25° C.

Table II

Effect of Temperature on the Growth of *P. megasperma* var. *sojae* on 20% V8/$CaCO_3$ filtrate agar

Days	Mean colony diameter in mm (N = 3)						
	5° C	10° C	15° C	20° C	25° C	30° C	35° C
2	0	0	12.3±0.8	15.2±0.3	20.0±1.7	16.2±1.3	0
4	0	8.7±0.5	20.0±1.0	27.3±1.0	39.3±3.2	33.2±3.3	0
6	0	10.0±0.0	29.5±0.9	43.3±1.5	58.7±5.8	51.8±4.4	8.5±0.5
8	0	10.0±0.0	40.5±1.3	58.3±2.0	78.8±7.0	70.7±4.0	9.7±0.3
10	0	10.8±0.3	52.3±1.3	75.2±1.8	99.2±6.7	88.5±3.5	10.5±0.9
12	0	12.8±0.3	66.5±1.3	92.8±2.0	121.2±6.3	108.3±3.8	11.5±0.5

Effect of temperature on mycelial dry weight

For liquid cultures, zoospores appeared to tbe the best inoculum by virtue of the negligible food materials they carry. It was found though, apart from the great chances of contamination, the mycelium developing from zoospores often stick to the bottom of the flasks so firmly that it was difficult to wash the mycelium out for dry weight determination. Subsequently, the following procedure was adopted. From the edge of an actively growing fungal colony on 20% V8/$CaCO_3$ filtrate agar (15 ml in 9-cm diameter petri dishes), discs were removed with a No. 2 cork borer and transferred to 15 ml V8/$CaCO_3$ filtrate in 250-ml Erlenmeyer flasks. The depth of solution was such that the agar disc was just submerged but there was no problem of aeration, due to the shallowness of solution. Mean dry weight of inoculum was determined by removing agar discs with mycelium into pre-weighed glass fiber pads and drying at 80° C. overnight. In order to overcome the great variations among replicates as indicated in preliminary experiments, 20 replicates were used per treatment. Due to the limited spaces in incubators, the experiment was conducted in three parts, mycelial dry weights being determined at the end of 5, 10 and 12 days respectively. Mycelium developed in each

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flask was filtered off using a glass filter pad in a Büchner funnel and dried at 80° C. Net increase in dry weight was obtained by subtracting the dry weight of the incoculum. The results are recorded in Table III.

Dry weight determination at the end of 5 days showed a similar growth/temperature relationship to that measured by colony diameter. The maximum growth was at 25° C. and was significantlty better than that at 15° or 30° C. (0.01 level). At the end of 10 days, maximum dry weight was at 20° and 25° C. with no significant differences between them but respectively greater than that at 15° C. (0.01 level) and 30° C. (0.05 level). At the end of 12 days, though the optimum appeared to be 15° C., it was found statistically non-significant when compared with 20° and 25° C. but growth at 25° was still significantly better than that at 30° C. (0.05 level). Probably, fungal growth reached maximum earlier at 25° and 20° and then autolysis developed rapidly. These results provided an example of the obscurity about the real concept of a

Table III

Effect of Temperature on Growth of *P. megasperma* var. *sojae* in 20% V8/$CaCO_3$ filtrate solution Culture

Temperature in C	Mean mycelial dry wt in mg		
	5 days (N = 16)	10 days (N = 19—20)	12 days (N = 15—19)
10	No growth	0.89± 0.89	4.26± 1.93
15	9.48± 4.26	38.35±15.31	65.70± 2.46
20	25.43± 7.71	51.29±11.82	62.45± 7.18
25	49.10± 7.43	50.10±10.85	60.42± 4.33
30	31.80±12.61	40.80±13.58	55.08± 7.99
35	No growth	1.07± 1.44	13.52±19.69

temperature-growth-optimum and the limitations of choosing only one incubation period (C o c h r a n e, 1958). Based on the definition that the optimal temperature is the temperature at which the fungus grows fastest during its actively growing period, the optimum for this fungus was 25° C., whether growth was measured in terms of colony diameter or increase in mycelial dry weight.

E f f e c t o f p H

The pH of liquid cultures was adjusted with N NaOH and N HCl respectively. Fifteen replicates were used for each treatment and the flasks were left a room temperature (24° to 25° C.). Mycelial dry weigths were determined at the end of 5 days. The results are recorded in Table IV.

The optimal range was between 6.2 to 6.6 with no significant differences in the fungal growth among various pH values within these limits. As for the other values below pH 6.2 and above 6,6, there were

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significant differences in mycelial dry weight between successive pH values with the exception of the pairs of pH 4.5 and 5.5; 7.0 and 7.4. The fungal metabolites tended to lower the pH of the medium as indicated by the final pH values at the end of the experiment. Nevertheless, since it took about 2 days before conspicuous mycelial growth started, and the experiment only lasted for 5 days, the initial pH values were considered more important. Suhovecky (1955) studied the effect of pH on the growth of this fungus on corn meal agar. He found two optima occurring at pH 6.0 and the second at pH 9.0. He admitted that this was an unusual case. Since different buffers were used to shift the pH towards the acid and alkaline sides respectively, his curious results might represent an example of chemicals on fungal growth.

Thanks are due to Dr. J. A. Haas, Harrow Expt. Station, Ontario, for supplying the culture and to Prof. C. J. Hickman, Univ. of Western Ontario for his constructive criticism.

Table IV

Effect of pH on the Growth of *P. megasperma* var. *sojae* in solution Culture

Initial pH	Final pH	Mean mycelial dry wt in mg (N = 9—15)
4.10	4.0	3.05 ± 1.18
4.50	4.40	17.70 ± 5.20
5.50	4.85	20.70 ± 4.10
5.80	4.90	28.37 ± 6.30
6.20	5.20	54.41 ± 10.01
6.25	5.30	57.30 ± 6.39
6.35	5.55	58.57 ± 7.61
6.60	5.60	59.89 ± 2.66
7.0	5.05	17.21 ± 2.48
7.40	5.45	15.31 ± 2.98
7.70	5.85	9.72 ± 0.96
7.85	6.20	7.47 ± 0.62

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Digitale Literatur/Digital Literature

Zeitschrift/Journal: Sydowia

Jahr/Year: 1970/1971

Band/Volume: 24

Autor(en)/Author(s): Ho H. H.

Artikel/Article: A Study on the Growth and Sporulation of Phytophthora megasperma var. sojae. 51-58