A PSYCHROPHILIC FUNGUS CAUSING GERMINATION FAILURE OF CONIFER SEEDS

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by

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SCOPE AND CONTENTS: The cause of germination failure in conifer seed beds at Midhurst and Orono Forest Nurseries was investigated. Attention was especially given to the isolation of a pathogen which kills - but does not rot - the seeds. Its relationship to the familiar damping-off fungi was clarified. Studies were also made of important biological functions of the pathogen. A practical method of controlling the disease was found.

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INTRODUCTION

In the Provincial Forest Midhurst and Orono Nurseries of the Ontario Department of Lands and Forests, the author observed in May and June 1957 areas in nursery seed beds devoid of plants. The beds had been sown in autumn 1956 with seeds of Pinus resinosa Sol. Sometimes these barren areas were large, approximately 25 square feet or more, sometimes only a square foot, or even less. Most frequently they were sharply demarcated from ajoining areas where seedlings were in abundance. The seedlings in the latter showed no evidence of disease. Occasionally, although very seldom, some seedlings were found in the barren areas. In the fall of 1958 at the Midhurst nursery, an area 40 x 50 yards was sown with Pinus resinosa seeds for a fertilizer experiment, and the following spring only scattered seedlings appeared. At least 95 percent of the viable seeds in this area did not develop seedlings. In 1960 another compartment, 50 x 200 yards, produced only 12 percent of plants from the viable seeds sown, according to data supplied by the nursery. In 1961, and especially in 1962 in the Midhurst nursery seed beds of Pinus resinosa, Pinus strobus L. and Pinus sylvestris L., barren areas were observed in very large number (Figs. 3 to 7).

The germination failure of conifer seeds observed by the author could be regarded as example of preemergence damping-off disease. Damping-off in conifers is a disease caused by fungi which are able to

attack plants from the seed stage to two year old seedlings. Descriptions of such a disease can be found as far back as 1795 when Schreger in his work "Erfahrungsmässige Anweisung zur richtigen Kenntnis der Krankenheiten der Wald und Gartenbäume", described the damping-off of beech seedlings. Hartley (1921) defined damping-off as a disease that results in a rapid decay of young succulent seedlings. It is caused by a number of soil inhabiting fungi that are facultative parasites and not specialized as to host. According to Hartley there are three main types of damping-off: (a) "Germination loss" - the radicles are killed soon after emerging from the seed coats and before the seedlings appear above the ground, (b) "Normal damping-off" - the still succulent seedlings are invaded by parasites at any point on the root or lower part of the stem, (c) "Late damping-off" - root infection of seedlings several weeks old. More recently the term "Germination loss" has been replaced with by "Preemergence damping-off" (Fisher, 1941). Hartley, (1918) discussing decay of dormant seeds writes: " .... to what extent coniferous seeds suffer from microorganisms before beginning to germinate can not be stated without further investigation". Since that time few references have been added to the literature dealing with germination loss of conifers. The most explicit work to date on germination loss of conifer seeds is that of Rathbun-Gravatt (13), but it is based on the influence of known fungi on seeds artificially inoculated. She used more than thirty different fungi and writes: "... Poor germination of pine, as a result of the work of damping-off parasites, is mainly due to the destruction of

radicles after they have emerged from the seed but before the seedlings are large enough to break through the soil. Decay of unruptured seed apparently can be caused by these fungi, but it appears to be of less importance as a cause of germination loss".

Subsequent investigation of barren areas mentioned earlier revealed that the seeds were slightly swollen, but otherwise appeared exactly as sown, the seed coats were unruptured, and the endosperm and embryo were light in colour. This is in contrast to "Germination loss" as described by Hartley where the seeds usually showed signs of germination and always were rotten.

In this thesis the author describes a study of the above germination failure of conifer seeds in which he has undertaken:

1. To determine why the areas of germination failure were usually sharply delimited from unaffected areas.
2. To isolate from the affected seeds a pathogen capable of producing the disease in artificially infected seeds.
3. To study the host range, distribution and spread of the pathogen.
4. To make cultural studies of the effect of environment on the pathogen.
5. To relate these observations to the development of the disease of in the nurseries.
6. To make recommendations regarding control of the disease based on these experimental results.

MATERIALS AND METHODS

Beginning in 1958 from Midhurst and Orono nurseries several hundred non-germinated seeds have been collected from Pinus resinosa, Pinus strobus and Pinus sylvestris. Generally the seeds have been collected the summer after sowing but in some cases even 4½ years after sowing. The seeds were sorted, the ruptured were removed and the remaining seeds kept in vials in the laboratory.

For isolation of the pathogen, the seeds were washed with sterile distilled water and the seed coats were sterilized by dipping in 1:1000 $HgCl_2$ solution for 4 to 5 minutes. After that the seeds were rinsed five times with sterile distilled water and were left in the last water for at least 20 minutes. Then the seeds were placed in a sterile Petri-dish under the dissection microscope, where, under sterile conditions, the seed coats were removed.

Cultural media employed were:

1. Peptone Dextrose agar (Johnson, 1957) modified by author,

Agar	20.0 gm	
KH_2PO_4 . . .	1.0 gm	
$MgSO_4.7H_2O$ . .	0.5 gm	
Peptone . . .	5.0 gm	
Dextrose . . .	10.0 gm	
Conifer seeds . .	20.0 gm	
Distilled water .	1000.0 ml	pH 5.8

The conifer seeds were ground in a porcelain grinder until they formed a homogeneous mass; then one liter of distilled water was gradually added and boiled for 20 minutes stirring all the time, and then while hot it was filtered through cotton. To the cooled liquid all the other materials were added and made up to one liter. The mixture was heated slowly while stirring until it boiled. It was then put in 250 ml flasks and autoclaved at 15 lb pressure for 20 minutes.

Peptone Dextrose agar was the medium used most frequently for isolations, and usually wherever a solid medium was needed. Sometimes the medium was employed in liquid form, lacking agar.

2. Czapek's sucrose nitrate agar (Conn, 1921):

Agar	15.0 gm	
$NaNO_3$	2.0 gm	
K_2HPO_4	1.0 gm	
$MgSO_4.7H_2O$	0.5 gm	
KCL	0.5 gm	
$FeSO_4.7H_2O$	0.01 gm	
Sucrose	30.0 gm	
Distilled water	1000.0 ml	pH 7.3

Czapek's agar lacking sucrose was used as the basic medium in experiments on the nutrient value of amino acids and some other substances.

3. Soil extract agar (Allen, 1957) modified by author,

Agar	15.0 gm	
Glucose	1.0 gm	
K_2HPO_4	0.5 gm	
Soil extract	100.0 ml	
Tap water	900.0 ml	pH 6.7

Preparation: Soil extract was prepared by autoclaving 1000 grams of conifer nursery soil with 1000 ml of tap water for 20 minutes at 15 lb pressure. The solution was filtered through double paper (Whatman #1) filter and made up to 1000 ml, then bottled and sterilized in 100 ml quantities. Soil extract agar was used sometimes for isolation attempts.

4. Yeast extract agar,

a.

Yeast extract	5.0 gm	
Agar	15.0 gm	
Distilled water	1000.0 ml	

b.

Yeast extract	10.0 gm	
Agar	15.0 gm	
Distilled water	1000.0 ml	pH6.6

Yeast extract is an excellent source of B-complex vitamins and is often used to supply these factors in culture media.

5. Malt extract agar,

Malt extract	30.0 gm	
Agar 15	15.0 gm	
Distilled water	1000.0 gm	pH4.7

6. Oxgall agar (Littman, 1947),

Agar	20.0 gm
Peptone	10.0 gm
Dextrose	10.0 gm
Oxgall	15.0 gm
Distilled water	1000.0 ml
Crystal Violet	0.01 gm

pH 7.0

Oxgall has been used in the preparation of a selective medium for fungi. Littman described oxgall agar for isolation of pathogenic fungi, employing oxgall, crystal violet and streptomycin as inhibiting agents for bacteria.

7. Corn meal agar,

Corn meal, infusion from	50.0 gm
Dextrose	2.0 gm
Agar	15.0 gm
Distilled water	1000.0 ml

For cytological studies, the fungus was grown on Peptone dextrose agar, but since it was hard to separate the hyphae from the agar without damaging them, a membrane (Millipore) culture method and Giemsa stain described by Miller (1959 and 1961), slightly modified, was used. Millipore membrane filter of type TV 20 u thick with pore size 50 uu was cut into pieces 5 x 10 mm. The pieces of membrane were sterilized by autoclaving in distilled water, placed on the surface of freshly made Peptone dextrose agar in the Petri-dishes beside the fungus inoculum and incubated at 15^{0}C or other temperatures in an incubator.

When the hyphae had overgrown half of the membrane filter, the filter was separated from the rest of the culture by cutting with a sharp scalpel. The staining procedure was as follows:

1. The membranes bearing the fungus hyphae were transferred with a fine-pointed forceps to a dish containing 0.5 percent aqueous sodium azide, for three hours (for cytoplasmic killing).
2. They were then transferred to 10 percent perchloric acid at 60°C for one minute.
3. The acid in the membranes was neutralized by treatment for 30 minutes with freshly prepared double-strength G.T. Gurr's pH 7.0 buffer.
4. To 7 ml of this buffer in a Petri dish was added 1.5 ml of G.T. Gurr's Improved Giemsa Stain, R66, mixed well and the membrane bearing the hyphae were transferred to the Petri-dish for one day at room temperature.
5. Excess stain was removed by repeating step 3.
6. The membranes were dried in the air by impaling the membranes on insect pins which were then placed vertically in a piece of paraffin wax. Then the membranes were transferred to isopropanol to ensure complete dehydration.
7. The membranes were mounted on a thin layer of G.T. Gurr's Neutral Mounting Medium on a microscope slide; the slide was covered with a box lid and allowed two days to dry. Then a small drop of the same medium was placed in the centre of each, a #1 coverglass was added, and pressed down gently. On top of the coverglass Whatman #3

filter paper was placed, covered with another slide and the whole was squeezed for one day with a book clamp.

The nuclei of the cells thus treated were deep blue, and the cytoplasm was light blue.

EXPERIMENTS

1. Isolation of a Psychrophilic fungus from non-germinated Pinus resinosa Sol. seeds

Non-germinated seeds whose external appearance was healthy were collected from seedling free areas of Pinus resinosa seed beds for use in an attempt to isolate a pathogenic microorganism. On opening 120 of these it was observed that 8 were empty, 7 were rotten, and the remaining 105 (87%) had, on the whole, light-coloured endosperms and embryos, that showed no indication of germination, yet appeared to be healthy.

In the first series of experiments the seeds were not surface sterilized but were washed five times with sterile distilled water. The washed sees were transfered to Petri-dishes (20-25 per dish) containing 30 ml Soil extract agar, Peptone dextrose agar or Malt extract agar. The Petri-dishes with the seeds were kept in a dark place in the laboratory, where the temperature varied from 18°C to 30°C. After 4 to 5 days all the seeds were covered with a large amount of fungus mycelium. Bacterial growth was also abundant. Common saprophytes including Penicillium, Aspergillus, and Mucor were so abundant that isolation of a pathogen from the dishes would have been exceedingly difficult. Two repetitions of this experiment gave the same result.

The experiment was again repeated employing as culture media

Peptone Dextrose agar containing Rose bengal (1:30,000) and Chlortetracycline (30 mcg/ml), and Oxgall agar containing Crystal Violet (1:00,000) and Chlortetracycline (30 mcg/ml). Rose bengal dye was found by Martin (1950) to slow down development of rapid growing fungi in soil platings and this facilitated the isolation of slowly growing soil fungi. Oxgall has a similar effect (Littman, 1947). Crystal violet dye and Chlortetracycline were included to reduce bacterial development.

Again the Petri-dishes with the seeds were placed in a dark place in the laboratory. The following day it was learned that it would not be possible to examine the plates in the immediate future, so to slow down growth of microorganisms they were placed in a cold room, the temperature of which varied between 1° and 3°C. After two weeks the plates were examined and it was found that fungus growth was present on all seeds and frequently bacteria also. This growth was always slight and rarely advanced more than a millimeter or two from the seeds. On many of the seeds there was an outgrowth of short thick colorless, transparent hyphae which did not grow into the agar. Material from those outgrowths was removed by using a pointed scalpel, transplanted to new agar plates containing the same medium, and kept at room temperature. There was no growth of the hyphae and within a week they were overgrown by other fungi.

In the next attempt at isolation the seed coats were removed because a pathogen capable of preventing germination should be inside the seed. Thus superficially contaminating fungi could be eliminated.

The procedure was as follows: the seeds were washed with sterile distilled water and transfered to a mercuric chloride (1:1000) solution for 1, 2, 3, 4, or 5 minutes. After that the seeds were rinsed five times with sterile distilled water and left in the last water for at least 20 minutes; they were then placed four to a sterile Petri-dish and under the dissection microscope, the seed coat and endosperm coats were removed using sterilized instruments. Naked endosperms were transferred to Petri-dishes with Peptone Dextrose agar without Rose bengal and Chlortetracycline, but with 5 percent (instead of the usual 2 percent) ground Pinus resinosa seed. Since the growth of transparent hyphae had been observed in the cold room, in this experiment, too, the Petri-dishes were placed in the cold room. After one month around the seeds there were hyphae like those described above but longer (2-4 mm in length). Seeds from this experiment are shown in figure 8-I and 9. Growth of other fungi or bacteria was very seldom observed in these Petri-dishes. Material from mycelium was transplanted to new plates containing the same medium and left at the same temperature (1 - 3^{o}C). After a month of growth small white fluffy colonies 2-4 mm in diameter were present.

During this work further isolations were often made using the foregoing procedure (surface sterilization, removal of coat, culture of Peptone Dextrose agar with 2 percent ground conifer seeds at low temperature, transfer to fresh medium), and the same fungus was consistently isolated in pure culture. For the sake of brevity, in this thesis it will be referred to as the S-fungus (seed fungus).

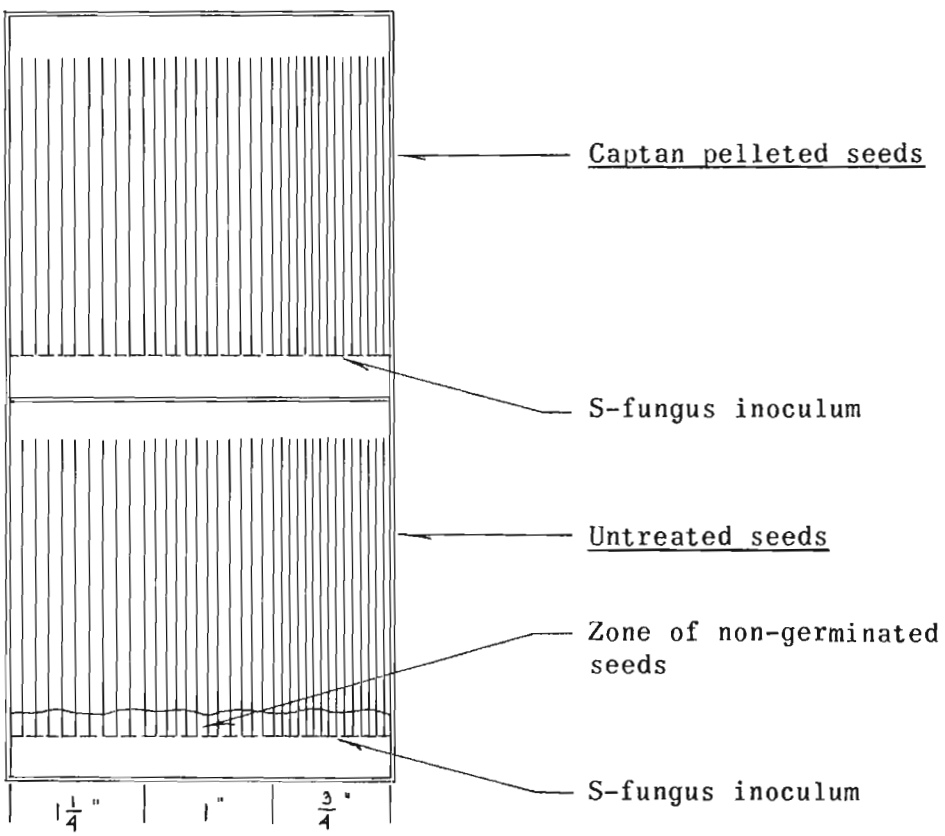
INFECTION EXPERIMENTS

2. Artificial infection of Pinus resinosa seeds with S-fungus

(Field experiment)

A seed bed was prepared by removing 4 inches from a bed 3' x 6'. The edges of the bed were strengthened with 1" x 6" wooden boards wrapped in aluminium foil. With the same type of board the bed was divided in half. This bed was filled with soil from the

FIG. 1 SEED BED FOR INFECTION EXPERIMENT



Midhurst nursery, which had been sterilized by autoclaving for one hour at 15 lb pressure. On November 8, 1960 the bed was sown with Pinus resinosa seeds; one section containing untreated seeds and the other seeds pelleted with Captan (Fig. 1).

They were sown ¼ inch deep in rows parallel to the longside of the bed; the distance between the rows was 3/4, 1, and 1¼ inches, and the distance from seed to seed in each row 5/8 inch. The rows started 4 inches from the edge of each section, one end of the rows was crossed by a row at right angles in which was placed inoculum of the S-fungus. This was prepared by autoclaving Pinus resinosa seeds in water in 2-cm test tubes, and inoculated with a piece of Peptone Dextrose agar on which the S-fungus was growing. After one month of growth at 18°C they were covered completely with the fungus. They were placed in the row in a single straight line close together and covered with the soil to a depth of ¼ inch.

In the spring of 1961, in the section where untreated seeds had been sown, beginning from the inoculum row for a distance that varied 2 to 3 inches, not a single seed germinated.

Using the method described above the S-fungus was reisolated from these non-germinated seeds. In the second section, where seeds treated with Captan had been sown, germination was regular and there was no evidence of disease. The distance between seed rows had no apparent influence on the final results.

3. Artificial infection of Pinus resinosa seeds with S-fungus

To 8 cm Petri-dishes Whatman #3 filter paper circles were added (2 per dish) and on the top of them were oven sterilized at 150°C for 10 minutes. 30 ml of sterile distilled water was added to each plate when it had cooled. In the middle of each plate was placed a 2 x 2 mm piece of S-fungus culture grown on Peptone Dextrose agar. Around the agar, in a star shaped fashion were placed 25 Pinus resinosa seeds (Fig. 13). Control plates were prepared in the same fashion but without the S-fungus. The plates with seeds were maintained at 1° to 3°C.

At this temperature the conifer seeds did not germinate, therefore, after two months, to determine whether seeds were still viable, the plates were transferred to 20°C.

After a period of three weeks at 20°C the seeds in the inoculated plates produced no germination. In three control plates two seeds did not germinate because of rotting, but all others germinated.

From the non-germinated seeds the S-fungus was reisolated.

4. Attempts to infect Pinus resinosa and Pinus strobus seedlings

Petri-dishes were filled with rich soil of high humus content, moistened with distilled water and autoclaved for 1 hour at 15 lb pressure. Each sterilized Petri-dish was inoculated in three different places from a culture of the S-fungus growing on Peptone Dextrose agar, and incubated at 18°C in an incubator. Seeds of P. resinosa and

P. strobus were sterilized according to the method described earlier, and germinated in the same incubator in the dark. When the seeds had developed radicles 1 to 1.5 cm long, which required about two weeks, the seedlings were transplanted to the Petri-dishes (4-5 per dish) in which the fungus had grown for 4 to 6 weeks and had covered the surface area. There were 4 Petri-dishes with P. resinosa and 4 with P. strobus seedlings. The radicles were placed in previously prepared holes in the soil keeping the seeds above the surface, and the soil was pressed in around the radicles. According to conditions the soil was moistened with sterile distilled water. In order to maintain maximum humidity the Petri-dishes were placed on a ground glass plate, several beakers containing distilled water were placed next to them, and Petri-dishes and beakers were covered with culture dishes 10 cm high. They were placed in a growth room 8 feet high and 9 feet by 12 feet in floor area in which illumination of 1600 f.c. was provided by fluorescent and incandescent lamps for 16 hours each day. The temperature was $18^{o}C \pm 0.5^{o}C$, but usually dropped when the light was off ($2-4^{o}C$). After ten days when needles had developed and the seed-coats had been shed, the same plants were reinoculated on the surface of the needles (by means of a forceps) with mycelium bearing a large amount of fungus spores.

On inspection of the plants forty days after the seedlings were placed in the soil or thirty days after the inoculation with spores, no signs of infection could be found. All the plants had developed side roots and additional needles, and no differences was evident between the

inoculated plants and the controls.

This infection experiment was repeated using quartz sand instead of soil (Fig. 12). The fungus hyphae grew much more thinly over the surface of the sand than over the soil. The seedlings developed very slowly and appeared weak because of deficiency of nutrient in the sand. This weak condition of the host together with the high humidity should have given optimum conditions for infection, but on examination over a period of three months no signs of infection could be found.

It may be concluded that the fungus was not able to attack P. resinosa and P. strobus when the seedling stage was reached even if the seedlings were grown in an environment (quartz sand) where development was poor.

5. Attempts to infect new shoots of Pinus resinosa and Pinus strobus

Two year-old plants of P. resinosa and P. strobus were placed in eight-inch pots in the summer of 1961. In the spring of 1962 the potted plants were transferred from the Midhurst nursery to the Maple Laboratory and were placed at the north side of the building. In this location the plants received only one half hour of morning sun, and the rest of the day they were in the shade. When the main shoots of the P. strobus had reached a length of between 6 to 8 cm, and the P. resinosa 10 to 12 cm, the still undeveloped needles in the middle sections of the main shoots of five P. strobus and five P. resinosa plants were folded back and behind them pieces of Peptone Dextrose agar on which the fungus was growing were inserted. Each main shoot was

inoculated in three places. In order to maintain moist condition and to prevent the inoculum from drying out the inoculated shoots were covered with plastic bags.

On opening the plastic bags after a period of two weeks no evidence of infection was observed, and no difference could be seen between treated plants and the untreated controls.

The experiment was repeated in April, 1963 with two-year-old P. resinosa plants in a growth room at constant temperature ($18^{o}C \pm 0.5$), and constant light (1800 f.c., 16 hours daily) provided by fluorescent and incandescent lamps, and about 70 percent relative humidity. However, the temperature often dropped 2-3^{o}C when the light was off.

Inoculation was done in the same manner as in previous experiments. At the end of three months there was no evidence of infection.

From the results it may be concluded that the fungus was not able to attack new P. resinosa and P. strobus needles while they were elongating, even at very high humidity.

MORPHOLOGY

6. Morphology of the S-fungus in culture and in the host

The cells of hyphae from young S-fungus colonies from Peptone Dextrose agar are cylindrical and vary greatly in length (10 - 350 μ) (Fig. 42 and 43). In the older cultures the cells are shorter and less uniform in shape. The S-fungus cells from non-germinated seeds usually are relatively short (10 - 35 μ) with narrowed ends and resemble yeast cells (Fig. 41).

The terminal cells of the hyphae appear to have homogeneous cytoplasm when unstained mounts are observed with the light microscope, and the others are more or less granulated. It was frequently observed that in the development of new cells at the hyphal tips the cytoplasm moved toward the tips from the older cells.

Generally the S-fungus grows on Peptone Dextrose agar at 18^{o}C and higher as a sterile mycelium, but in certain cases on Peptone Dextrose and other media at a temperature of 15^{o}C it develops spores. The cells of the hyphae bearing spores are not different in structure from those of the vegetative hyphae. Conidiospores consisting of one or more cells variously arranged develop directly from the mycelium (Fig. 40, 45-52). Sometimes the conidiospore is separated from its mother cell by a cross-wall, but when it consists of only one cell it usually is without a cross-wall. The new spore is formed through the

narrowing of the end of the cell (Fig. 45). When the new spore has developed the conidiospore continues to grow and then another spore develops, the first remaining on the side (Fig. 46). Spores formed in this manner look like a cluster of grapes. The shape of the individual spore is rounded at one end and more or less conical at the other (Fig. 53). The conical end is not pointed but truncated.

Spores germinate without distinct germ tubes. First the spore begins to grow in length from the conical end. On reaching approximately 30 to 40 μ it becomes separated into two cells by a cross-wall and both continue to grow in length and become much longer and narrower. A new cell begins to develop also from the rounded end of the spore (Fig. 58). Gradually side branches are formed and the spore region disappears among the new cells.

The spores contain one nucleus, but the vegetative cells contain 15 to 20 nuclei. This was determined by Giemsa staining.

When autoclaved conifer seeds are inoculated by the S-fungus in and upon them, sometimes on the surface of the seeds the fungus develops a very compact mass of pseudoparenchymatous cells. These structures could be primordia for sclerotia development but in the culture tubes they do not develop further and their function is not clear (Figs. 14 and 42).

After growth for at least one month at 10 to 18^{o}C on Peptone Dextrose agar containing 2 percent by weight of conifer seeds, sclerotia sometimes develop. These are slightly greenish in colour, and irregular in form with either smooth or velvety surfaces from which

a few hyphae can be seen spreading out. On the other media employed in this study sclerotia did not develop.

In Petri-dishes and in 20 mm culture tubes it has been possible to obtain sclerotia up to 15 mm in width; the commonest size was 2 to 4 mm. Attempts to promote their development into fruiting structures have been unsuccessful. Treatments employed in this connection were:

1. Storage at a temperature of 1 to 3°C for a four week period.
2. Storage in the deepfreeze at -15°C for a four week period.
3. Allowed to dry out in an agar plate at room temperature.

After these treatments the sclerotia were transplanted to Peptone Dextrose agar containing 2 percent conifer seeds, the sclerotia then developed hyphae and formed new colonies, but no fruiting structures formed.

PHSYSIOLOGICAL AND NUTRITIONAL STUDIES

7. Growth of the S-fungus on different culture media

The S-fungus grew reasonably well on many culture media and the medium used influenced the colony form. The temperatures at which the cultures were grown also affected the form of the colony. Thus at low temperatures (circa 10 to 15°C) the colony growth was fluffy and white. At high temperatures (circa 25°C), the colony was dense and leathery, and was either yellow or yellow brown in color. The same color was obtained if growth took place in light, at 15°C.

Peptone Dextrose agar containing conifer seeds,

The S-fungus grew as a flat, round, silky colony, sometimes more or less fluffy. Cultures of the fungus freshly isolated from non-germinated seeds grew in a compact fashion, but after several months in culture they became more fluffy and grew more rapidly; this is probably due to the selection of a mutation or mutations which grow more rapidly in artificial culture than the original type. After a period of growth of two to three months on the same dish of medium at 18 to 20°C the culture became yellowish, yellow brown or brown (Fig. 17).

Corn meal agar,

The colonies were not so compact as on Peptone Dextrose agar.

Fewer hyphae developed and there were more aerial hyphae. From the margin of the colonies, several sectors usually grew (Fig. 18), but the form in general was circular.

Malt extract agar.

In comparison with foregoing media, the hyphal net was relatively thin. Usually around the inoculum it formed a small compact colony (probably due to the using up of nutrient in the inoculum) form which grew scattered hyphae which branching was not regular, and produced sectors containing thicker and thinner netting of hyphae (Fig. 19).

Czapek's agar,

The colonies had relatively thin hyphae net, the same size (4 to 5 cm) as that produced on malt extract agar but without sectors.

Czapek's agar without sugar,

Growth was very weak and apparently continued only while the fungus was able to utilize the food remnants in the inoculum (Fig. 22).

Czapek's agar without sugar and $NaNO_3$,

Practically no growth (about 1 mm).

Czapek's agar without sucrose but with 1 percent D-glucose,

The colonies had thicker layers of hyphae as than produced with sucrose.

Czapek's agar without sucrose and $NaNO_3$ but with $(NH_4)_2SO_4$, (0.15 percent),

A fluffy colony grew around the inoculum (7 to 9 mm) from which grew a few hyphae and branched at the edge of the plate.

Czapek's agar without sucrose but with 1 percent D-maltose, D-mannitol, D-lactose or soluble starch,

All these carbon sources produced colonies with scattered hyphae. Frequently such hyphae rapidly overgrew the whole. In same places thick clusterings of hyphae were found. Growth from the point of inoculation to the edge of the dish was about equally rapid with maltose, mannitol and soluble starch. With lactose fluffy hyphae grew around the inoculum (6 to 8 mm), and from them only a few hyphae grew to the edge of the Petri-dish and branched off. Long sections of hyphae were blue in color.

Czapek's agar with Nicotinamid (1 to 1000) but lacking sugar,

Small colonies developed (Fig. 20). The greatest amount of hyphae grew in the agar and the number of aerial hyphae has very small. The whole mycelium with the exception of the aerial hyphae became dark blue.

Czapek's agar plus 1 percent egg albumin instead of sugar,

Fluffy colonies formed from which individual hyphae grew and frequently formed branches.

It is characteristic that all mentioned additions to Czapek's

agar with the exception of sucrose, glucose and nicotinamide, facilitated the growth of individual unbranched hyphae, which tended to grow from the center of the Petri-dish where the inoculum was placed, progressed relatively quickly, and branched along the edge of the plate.

Quartz sand as substratum for growth,

Usually only three or four hyphae grew out from the inoculum and some developed spores though not so rapidly as Czapek's with soluble starch (above). The process can best be observed on clean, washed quartz sand in Petri dishes. It was frequently observed that in the development of new hyphal tips the cytoplasm moved toward the tips from the older cells. In beginning cultures there were only a few empty cells but in the later phases these could be found in large quantities.

Oxgall agar,

No growth.

Natural media:

Rich soil of high humus content,

In Petri-dishes containing 30 to 40 ml of sterilized soil the S-fungus grew rapidly, and after a month the soil was so heavily interwoven with hyphae that it tended to hold the soil particles together. When the Petri-dishes were placed under the bell jar and the humidity was enough, fluffy hyphae developed on the top of the soil.

Buds, needles and small branches as substratum for growth.

In order to determine the ability of the S-fungus to grow on various natural substrata the following material was collected:

Pinus strobus L. (White pine) - branches and needles,

Pinus resinosa Sol. (Red pine) - branches and needles,

Pinus pungens Lam. (Mountain pine) - branches and needles,

Picea glauca (Moench.) Voss. (White spruce) - branches and needles,

Larix decidua Mill. (European Larch)- branches with buds,

Betula populifolia March. (Gray birch) - branches with buds,

Populus balsamifera L. (Balsam poplar) - branches and buds.

Two experiments were done, the material for which was collected in March and February 1963. The branches used were 5 to 8 mm in diameter, and the needles were those formed during the previous year. The branches were cut in 7 to 8 cm lengths and split in two halves. The split branches were placed on end in the culture tubes (18 cm long and 2 cm in diameter) to form a single layer at the bottom of the tubes (Fig. 35). The needles were also placed on end to form one layer at the bottom of the tubes except for the Picea glauca needles which were placed in the tubes to a height of 6 cm. Three culture tubes were used for each material. Distilled water was placed in the tubes to a height 2/3 that of the material occupying the tubes. The tubes were sterilized by autoclaving at 15 lb pressure for 20 minutes. After the culture tubes had cooled down they were each inocultated with a piece of S-fungus which had grown on Peptone Dextrose agar. To prevent dehydration the tubes were covered with

parafilm and incubated at 20°C in an incubator for the first experiment and at 15°C for the second.

After two months the tubes were examined, with the following results:

Pinus strobus branches - there was a great abundance of hyphae on the exposed wood extremely on the ends, but less on the bark.

P. strobus needles - completely overgrown with hyphae.

P. resinosa branches - a great abundance of hyphae on the wood, in some areas the hyphal network was extremely thick.

P. resinosa needles - completely overgrown with hyphae.

P. pungens branches - both wood and bark overgrown with a thick coating of fungus hyphae.

P. pungens buds - a few hyphae around the buds.

P. pungens needles - a few hyphae on upper section of needles.

Picea glauca branches - completely overgrown with hyphae but fewer on the bark.

P. glauca needles - no growth.

Larix decidua branches with buds - both branches and buds were covered with hyphae.

Betula populifolia branches - there was a great abundance of hyphae on the wood but less on the bark.

B. populifolia buds - no growth.

Populus balsamifera branches and buds - no growth.

8. Influence of pH on growth of S-fungus

In order to study the growth of the fungus at different pH's, Peptone Dextrose solution containing 0.05 M phthalate or phosphate buffers were prepared and placed in 125 ml Erlenmeyer flasks, 40 ml per flask. They were sterilized for 15 minutes at 15 lb pressure and inoculated with the S-fungus in the same manner as in the temperature experiment (above). The flasks were kept in an incubator at 20°C. After twenty days the mycelium was removed, washed, dried and weighed in the same manner as in the temperature experiment. For each pH four flasks were used.

The results, shown in Table I, indicate that the optimum pH for growth is 6-7, but the fungus is capable of good growth over wide pH range, i.e. at least from 4.3 to 8.0, and this exceeds the usual pH range in conifer nursery soils in Ontario. The acidity of the soil in most nurseries is pH 5 to 7, occasionally higher, very rarely lower. Hence it is not likely that pH would limit the development of the fungus in nursery soils.

Table I

pH of medium after sterilization, at the end of experiment, and Mycelium weight per flask

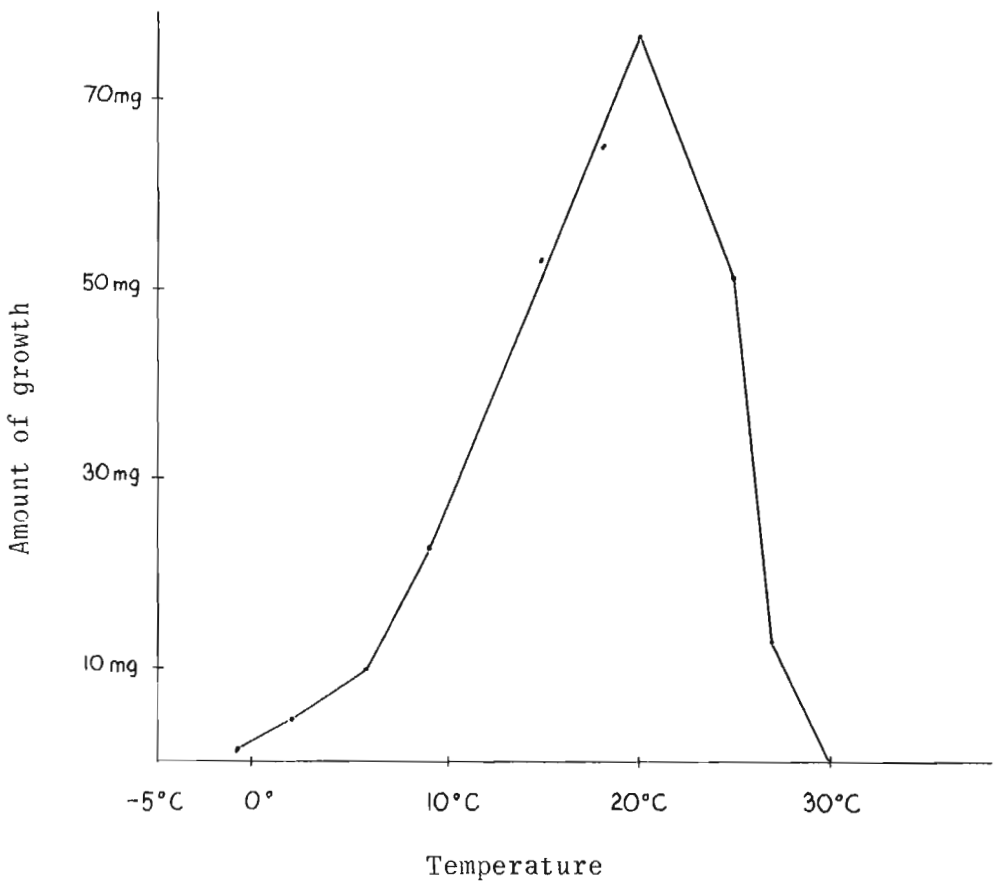
Buffer	pH of medium		Average Mycelium weight per flask in mg
	After sterilization	At the end of experiment	
Phthalate	4.1	4.3	6.5
Phthalate	5.0	5.2	17.2
Phosphate	6.0	6.2	18.6
Phosphate	7.0	7.0	18.6
Phosphate	8.0	7.6	15.1
Phosphate	8.7	8.0	14.9

9. Effect of temperature on growth of S-fungus in liquid medium

In 125 ml Erlenmeyer flasks, Peptone Dextrose solution was added (40 ml per flask) and autoclaved for 15 minutes at 15 lb pressure. For inoculum, S-fungus colonies grown for 10 days at 18°C on Peptone Dextrose agar were cut in 1 x 1 mm squares and these were distributed over the surface of the same medium so that the hyphae would develop on the sides of the pieces of agar. The advantage of allowing this development of hyphae was that when the pieces of inoculum were transferred to the flasks they remained on the surface of the liquid medium. The age of the inoculum when placed in the flasks was three weeks. To prevent dehydration the flasks were covered with Parafilm. The flasks were

placed in incubators at nine temperatures: -1, 1.8, 6.5, 9, 15, 18, 20, 25, 27 and 30°C. There were four flasks at each temperature.

FIGURE 2. Effect of temperature on growth of the S-fungus in liquid medium.



The results (Fig. 2), showed that the fungus did not grow at 30°C, and grew best at 20°C. It produced a small amount of growth at -1°C.

10. The effect of different temperatures and infection methods on germination of seeds and development of spores.

The Petri-dishes were prepared in the same way as in the previous experiment. The sterilized plates were divided in three groups, ten plates per group. In the first group rectangular pieces of agar 1.2 x 2 cm from a growing S-fungus culture were placed in the middle of the Petri-dishes. 20 Pinus resinosa seeds were placed around the agar in such a manner that each lightly touched it.

In the second group 20 seeds of P. resinosa were arranged in the middle of the Petri dishes and small pieces of agar from the S-fungus cultures were placed among these seeds.

In the third group, as in the second the seeds were put in the middle of the Petri-dishes but among them was placed S-fungus mycelium without agar.

In all cases the fungus was grown on Peptone Dextrose agar. The control plates were prepared in similar fashion but without material from S-fungus cultures.

Each of the above mentioned groups were divided among following temperatures: 4, 6.5, 12, 15 and 18°C (two control and two plates from each group at each temperature).

At 18°C all seeds germinated in 10 days (the seed coats were covered with S-fungus mycelium but the new seedlings dropped the

mycelium together with seed coat), but at the temperatures below 18°C they did not germinate in three weeks, even in the non-inoculated control.

TABLE 2

The effect of different temperatures and infection methods on germination of seeds and development of spores

Temperature °C	Group	Seeds			Spores	
		Germination %	Non-germinated seeds		Over seeds	Over quartz sand
			Non-rotted	Rotted		
18	I	100	-	-	-	-
	II	100	-	-	-	-
	III	100	-	-	-	-
	*C	100	-	-	-	-
15	I	92.5	5	2.5	many	few
	II	-	100	-	very many	many
	III	-	95	5	many	very many
	C	97.5	-	2.5	-	-
12	I	100	-	-	many	many
	II	100	-	-	many	many
	III	5	95	-	many	many
	C	100	-	-	-	-
6.5	I	100	-	-	many	many
	II	100	-	-	many	many
	III	95	5	-	few	many
	C	100	-	-	-	-
4	I	90	5	5	many	many
	II	95	5	-	many	many
	III	90	5	5	few	many
	C	95	-	5	-	-

*C = control

In order to determine whether the seeds were still able to germinate they were then placed at a temperature of 18°C for three weeks. The results are shown in Table 2.

The only severe germination failure occurred at 15° and 12°C indicating an optimum temperature for infection in this region but it was not found with all inoculation methods. The impression is obtained that if the S-fungus is applied in conditions where there is a considerable amount of desirable medium, it does not attack seeds quite as readily as in conditions where the medium is found only in small quantities or not at all.

The S-fungus developed spores only between 4 - 15°C but did not develop them at 18°C. This agrees with results of other experiments where the S-fungus was grown on agar media or on quartz sand at 18°C or higher temperatures but no spores had been observed. Likewise, no spores were obtained on such media at 1 - 3°C.

11. The influence of different amino acids as extra nutrient sources on growth of S-fungus

As indicated above the S-fungus develops well when conifer seeds are added to the medium. Conar (9) found in Pinus roxberghie Sa. endosperm and embryo by chromotography twelve amino acids, and two amides. The embryo and endosperm showed the same kinds of free amino acids at different stages of development. Bartels (1) in Pinus nigra Arn. Pinus sylvestris L. and Pices abies Karat. has identified eleven amino acids.

In order to determine which amino acids can be utilized by the fungus an experiment with twenty amino acids was designed, seventeen found in conifer seeds by Conar and Bartels, plus beta Alanine, DL Isoleucine and Tryptophane.

Czapek's agar without sugar but containing nitrate was used as the basic medium. The prepared agar was divided into parts and one amino acid was added to each part at the concentration of 1 gram per liter. Then it was sterilized for 15 minutes in the autoclave at 15 lb pressure. The sterile agar was poured into Petri-dishes. The Petri-dishes were then inoculated with the S-fungus cultures as described in the preceding temperature experiment and placed at $10^{o}C$ for ten days, and then at $15^{o}C$. Measurements of the colonies were made after ten and forty days.

The results are shown in Table 3. Sixteen of the amino acids used in the experiment supported the development of the S-fungus. Development of spores was facilitated by seven amino acids. Blue hyphal development was caused by three amino acids and fluffy colonies by three others.

TABLE 3

The influence of different amino acids as nutrient sources on the growth of S-fungus

	Amino acids	Czapek's agar without sugar but containing nitrate			
		Measurements of colonies in mm		Spores after 40 days	Remarks
		After 10 days	After 40 days		
1	L (-) Alanine	32 x 32	70 x 70	many	
2	beta Alanine	0	5 x 5	0	Blue hyphae
3	L (-) Arginine Monohydrochloride	30 x 31	52 x 54	0	Thick colony with undulate margin
4	DL Asparagine	22 x 24	30 x 34	0	Thick colony
5	DL Aspartic acid	12 x 12	15 x 15	0	Occasional blue hyphae
6	L Aspartic acid	21 x 22	90 x 90	many	Close colony at 10^{o}, scattered hyphae at $15^{o}C$
7	L (-) Cystine	4 x 4	6 x 6	few	Occasional hyphae
8	DL Glutamic acid	20 x 20	36 x 36	many	
9	Glutamine	24 x 26	36 x 38	0	Thin colony
10	Glycine	0	0	0	
11	DL Histidine HCl (Dihyd)	5 x 5	10 x 10	0	Occasional hyphae
12	DL Leucine	11 x 11	28 x 30	few	Fluffy colony
13	DL Isoleucine	18 x 18	26 x 26	0	
14	DL Phenylalanine	12 x 14	20 x 26	0	Radial undulate col.
15	L (-) Proline	25 x 30	30 x 36	0	Very fluffy colony
16	DL Serine	4 x 4	5 x 6	0	
17	L (-) Threonine	8 x 8	13 x 13	0	Fluffy colony
19	DL Tyrosine	7 x 7	10 x 12	few	
20	DL Valine	0	8 x 8	0	Few blue hyphae

12. Characteristics of the pigment in the cells of the S-fungus

Usually cultures of the S-fungus appeared as a white mycelium with a silky gloss (Fig. 11). At higher temperatures of growth such as 27°C the colour was yellowish. In very old cultures the colour was yellow-brown. Apart from this, a bluish colour was sometimes observed, when cultures growing on water agar were kept at a temperature of 1 to 3°C. The colour was first noticeable in places where cells join one another. In older cultures the whole cell was coloured and the coloured cells were separated by cells lacking pigment (Fig. 36 and 38).

The brightest cell colouring was observed when the fungus was grown on Czapek's agar plus nicotinnamide (0.2%). In this environment the whole colony with the exception of the aerial hyphae (which always remained colourless) became dark blue. Generally the blue hyphae were also a little thicker than the colourless. Coloured hyphae have also been observed in cases where the colony of S-fungus was growing together with other fungi, such as Penicilium sp. At the border between both colonies S-fungus became blue in colour. On transplantation of these hyphae to Peptone Dextrose agar, the colourless cells developed side branches and growth was resumed, but the blue cells were not seen to develop branches. Gradually the colour disappeared from the coloured cells of the inoculum. If hyphae consisting of only blue cells were inoculated, growth was not resumed.

DISCUSSION

Ever since seeds have been sown plant growers have known that not all of the seeds will germinate and produce healthy seedlings. In forest nursery practise in order to estimate how many seeds will germinate seed extraction stations conduct germination experiments and in such a way it is possible to determine how many seeds are needed for what area to obtain the required number of plants. But the calculations are not always correct, since the estimated percent for germination applies only to the seeds germinated in optimal conditions. In conifer nurseries where plants are grown in the millions, the germination of seeds and the development of new seedlings is determined, to a greater or lesser degree, by various diseases which may attack still non-germinated seeds, seeds in the process of germination, or just barely germinated seeds in which the new seedlings are destroyed before they emerge from the soil.

In this thesis the author has described a study of a disease which kills the seeds without rotting them before the seeds start to germinate. Low temperature has proven beneficial for the isolation of this unkown fungus from non-germinating seeds collected in nurseries. This is possible since the low temperatures prevent the development of rapid growing fungi which takes place at higher temperatures. To further prevent the influence of other fungi the seeds should be sterilized and the coat removed. For the sake of

simplicity the isolated fungus in this paper is refered to as the S-fungus (seed fungus). In favourable conditions the S-fungus is capable of destroying sown seeds 100 percent.

In forest pathology all diseases affecting seeds or young seedlings are referred to as damping-off. "Damping-off is a term applied to any disease that results in the rapid decay of young succulent seedlings or other shoots. It is caused by a number of soil-inhabiting fungi that are facultative parasites and not specialized as to host" (Hartley 1921). Boyce (1961) in his book "Forest Pathology", defined damping-off as "fungous invasion leading to the early decay and death of seedlings whose stems are still soft and succulent". Damping-off fungi spread through the soil and infect the seedlings by penetration of the epidermis. Two stages of the disease may be recognized: (1) "preemergence damping-off, in which the damping-off organisms decay the seeds or kill the seedlings before they emerge from the soil" (Fisher 1941); and (2) postemergence damping-off, in which the seedlings are affected after they appear above the ground. Killing of the roots and tops of seedlings by damping-off fungi may even be found in seedbeds during the second and even into the third season.

The disease has world-wide distribution and a wide range of hosts and in its various forms, is generally the most destructive disease in conifer nurseries. All common genera of conifers are susceptible and are known to be infected with the exception of the cedars which show a resistance. But even with the susceptible genera

there will be great variation in susceptibility in different localities, depending on the fungi encountered, nursery soils and climatic conditions. Losses at many nurseries are regular or iregular. In one season all the seedbeds of some species may be destroyed, but the following season damage may be unimportant. The damping-off fungi live saprophytically in the upper layers of the soil, and under favorable condition become pathogenic. Many fungi known to be causal agents of damping-off and the major groups are: Phycomycetes - Pythium spp. and Phytophthora spp.; Fungi Imperfecti - Rhizoctonia solani Kühn, Botrytis cinerea Pers., Fusarium spp., Sclerotium bataticola Taub, Diplodia pinea (Desm.) Kickx, Cylindrocladium scoparium Morg., and Pestalozzia funerea Desm.

The most important condition which promotes the growth of damping-off fungi are: high soil temperatures for certain fungi and low for others, high humidity and pH above 5.2.

Control is very difficult, because damping-off is caused by a number of different fungi and there is not a constant relation between environmental factors. Various fungicides give some protection to the seedlings but have various effects on different fungi.

The custom of calling all seed diseases damping-off is not well founded. To that respect we find but few commentaries in the literature. For example Fisher (3) writes: " ... there is reason to suspect, however, that seeds are decayed by organisms other than those known to cause damping-off".

The S-fungus does not belong to the damping-off fungi group.

It does not cause the decay of radicles nor decay of other parts of new succulent seedlings. Under laboratory conditions the S-fungus does not cause root rot and top killing which has been observed to be caused by several of the damping-off fungi.

The S-fungus is able to grow at -1 to +27°C but does not grow at +30°C. Usually organisms growing at a low temperature are commonly called psychrophiles but the exact definition is an unsettled problem. Considerable disagreement exists among the published definitions. Lamanna and Mallette (1959) defined psychrophiles "as organisms with a low maximal temperature for growth, say 20 to 30°C, if they are obligate psychrophiles, and which are also able to grow at low temperatures". Ingraham and Stokes (1959) state "Psychrophiles have been defined as organisms which grown appreciably and often abundantly at 0°C within 2 weeks. They can develop below 0°C and the minimum temperature is close to -10°C". But Roth and Wheaton (1962) write about the genus Arthrobacter "There appeared to be a continous gradation among members of this genus in ability to initiate and maintain growth 0°C".

The S-fungus in its growth at different temperatures would seem to meet the requirements for classification as a psychrophile.

In general practise in forest nurseries it is rather difficult to determine what percent of seeds germinate in seed beds since the seeds do not germinate simultaneously; while the last seeds are still in the process of germination, the seedlings from the seeds germinating first may already have become infected and been destroyed.

Even if the number of new plants is most carefully controlled, it still does not indicate how many seeds had germinated. It is practically impossible to determine what percentage of the seeds that did germinate were killed in their seedling state, before they emerged from the soil.

A question arises as to why other investigators who have done research in the field of seed non-germination have not discovered the S-fungus. Several reasons can be suggested: (a) chief attention is usually paid to those seeds which germinate and then show disease symptoms. The infection is clearly visible and easy to find. Less attention is paid to seeds which do not germinate and are not rotten since it is quite possible that they would be incapable of germination under any condition possibly due to the fact that the embryo has not developed.

(b) Damping-off fungi are so widely distributed that they can be found in practically all nurseries and the seed coats of all non-germinated seeds usually are contaminated with damping-off fungi. If such non-germinated seeds are placed in a culture dish, in a short period of time the seeds get covered with a large number of hyphae from which a pure culture of damping-off fungus can be isolated. It appears that with the isolation of a known damping-off fungus the problem is solved since it seems that the cause of the disease has been discovered. But the S-fungus as a slow grower gets covered up and remains unobserved.

(c) In order to isolate microorganisms from non-germinated seeds the

general process is to place the seeds on the agar in the Petri-dishes at room temperatures. In a laboratory in the summer the temperature usually exceeds 20°C which may be too high for development of the S-fungus but is well suited for the growth of other fungi.

(d) Since they usually attempted to isolate all the fungi within a seed (seed coat included) the seed coat is not removed and provides inoculum of other fungi which grow usually more rapidly than the S-fungus.

The microtome section of the non-germinated seeds indicated that endosperm and embryo cells are interwoven with the S-fungus hyphae; it is almost impossible to find host cells without hyphae either in the endosperm or the embryo. How the actual infection of the seeds does take place is still an unanswered question. In the studies of endosperm and embryo cells it was observed that the S-fungus hyphae in development following infection invade the cells at any place.

Experiments indicate that to kill the seeds a relatively long time is required (approximately a minimum of three weeks). A longer time is needed at the lower temperatures. One may think that the seeds become infected in the autumn, after sowing, when the soil temperature is still sufficient for the development of the S-fungus which grows until the earth is frozen or, in regions with a warmer climate it could grow the whole winter. If snow covers the ground before the earth has frozen, the temperature of the soil may remain

in limits suitable for growth of the S-fungus. In the spring the S-fungus may continue its growth immediately after the earth has thawed until the temperature for seed germination has been reached.

Laboratory experiments showed that the S-fungus develops very favourably in soil with humus content. The humus soil is interwoven by the S-fungus hyphae with a thick hyphae net and they develop rather evenly in all directions from the point of inoculation. This symmetrical growth as well as the fact that all seeds coming in contact with the S-fungus are destroyed, is most probably responsible for the empty areas in the seed beds with their sharply defined borders. Very often they are circular in shape which is less frequently observed in the cases of regular damping-off.

Laboratory observations that the S-fungus develops favourably in humus rich soil are supported by findings in nurseries that after humus was given germination usually was very low. For example autumn sowing of red pine seeds in compartment A4 at Midhurst nursery received a large amount of forest duff. In the following spring the germination was 2 percent or less. Others, compartments too, where non-germination of seeds was observed, from time to time have received relatively large amounts of humus - as farmyard manure or in later years as peat (about 45 cu. yds./acre).

pH, which considerably influences the spread of damping-off, does not to the same extent influence the S-fungus which develops reasonably well at pH 4.3 and pH 8.5. Optimum pH for the S-fungus development is 5 to 8. The acidity of the soils in most Ontario

nurseries is pH 5 to 7, occasionally higher, very rarely lower. Hence, it is not likely that pH would limit the development of the fungus in Ontario nurseries.

The S-fungus is capable of maintaining itself in the soil for a very long time especially if it is present in dead seeds. According to the observations of the author the infected non-germinated seeds are sometimes washed out from the seed beds in rainy periods and together with various humus particles may be deposited in lower areas among the beds, where the still non-germinated seeds are subjected to the heat of the summer sun and the temperature which sometimes exceeds even 40°C. During rainy periods such areas are flooded, followed by dryness. And yet even in such conditions the S-fungus may be found in a visible state in the dead seeds after a period of four and a half years (after sowing) and, most probably this condition may continue longer.

From pathological point of view the fact that the S-fungus may exist in infected seeds for such a length of time is a very important one, since the sowing of conifer seeds in the same spot may be usually repeated after 4 to 6 years. Even if the intervening period between sowing of conifer seeds were unfavourable to the growth of the S-fungus then it has to be realized that the formerly infected seeds serve as inoculum reservoir. Hence, when sowing the conifer seeds in previously used and S-fungus infected plots, even after a period of several years, it is very likely that, given proper conditions, the possibility of infection will be good. Due to the

above factors, in the fight against the S-fungus the usual plant rotation would not be expected to give positive results because this pathogen may for years survive in the soil showing no signs of activity.

Control. Since the S-fungus attacks only seeds they must be protected till they start to germinate. This is possible through pelleting the seeds with Captan [N-(trichlor methylthio) - 4-cyclohexene -1, 2-dicarboximide]. Seeds treated in such a manner are protected from the S-fungus until the time of germination. By the same token the process of pelleting, more or less, protects also against damping-off fungi.

Other fungicides have not been tried since seeds pelleted by Captan were available at Midhurst nursery where it was used to reduce the damping-off.

With the finding and recommendation by the author that Captan pelleted seeds are protected from the S-fungus, already from 1960 on, at Orono nursery all seeds were pelleted, and at the Midhurst nursery the same was started in 1962.

The S-fungus has been isolated from non-germinated seeds of Pinus strobus L., Pinus resinosa Sol., Pinus sylvestris L. and Picea glauca (Moench.) Voss. but in laboratory experiments it infected also Pinus banksiana Lam. and Picea mariana (Mill.) B.S.P. and Picea abies Karst.

In conifer nurseries the S-fungus can cause great damage. In personal discussion with the superintendant at Midhurst,

Mr. Halpanny the latter emphasized that even in white spruce (Picea glauca) beds, prior to using pelleted seeds, the level of germination had been very low and in several instances they obtained as little as 15 percent of one year plants from the number of visible seeds sown. In 1960 in compartment A13 (Midhurst) non-pelleted white spruce (Picea glauca) seeds produced one year old seedlings 70 percent less than pelleted seeds in the same compartment. Non-pelleted scotch pine (Pinus sylvestris) seeds in compartment A37 in 1962 germinated only 2 to 5 percent (isolated the S-fungus). In compartment A3 in 1960 non-pelleted red pine (Pinus resinosa) seeds produced only 12 percent one year seedlings (isolated the S-fungus). Red pine in compartment A4 (experimental part) non-pelleted seeds in 1959 produced only approximately 2 percent seedlings (isolated the S-fungus). In other compartments also, where non-pelleted seeds had been used, the germination percentage had been low. The same seeds pelleted and sown in the same or other compartments germinated well.

With the isolation of the S-fungus and established preventative methods the nurseries have the opportunity to renew seed beds, in areas where due to the presence of the S-fungus the seeds appeared to be a complete loss. With a smaller number of the seeds sown, the nurseries can grow the same number of the seedlings at considerable saving.

The ability to protect against the S-fungus through pelleting the seeds gives a much clearer understanding of conifer seed protection.

In practical application a number of authors, working with the same fungicide have returned different findings and it has been very difficult to explain these differences (Vaartaja 26).

If we find that in a given nursery the S-fungus is abundant, pelleting should provide a great improvment in the germination of seeds. If for another example, in a given nursery the S-fungus is not present or very scarce, but damping-off fungi abundant, pelleting may be less effective.

The find that the S-fungus is able to survive many years in seeds in active form is of practical importance in the laboratory because this provides a convenient method of S-fungus preservation (there is no need for frequent transference to new media). Also there is less chance for development of new strains of the organism which happens so often in laboratory cultures maintained by serial transfer.

SUMMARY

It had been observed for several years that at Provincial nurseries located at Midhurst and Orono (Province of Ontario-Canada) in certain compartments germination of seeds was very low. In extreme cases seed germination reached only 2 percent.

Between 1959 and 1963 from above mentioned nurseries non-germinated seeds were collected from beds of Pinus resinosa Sol., Pinus sylvestris L., Pinus strobus L. and Picea glauca (Moench.) Voss. From all collected seeds it was possible to isolate an unknown psychrophilic fungus which grows between -1 to +27°C but does not grow at 30°C. In seed beds in infected dead seeds the fungus was found in the viable state after a period of four and a half years where the temperature sometimes exceeds even 40°C.

This unknown fungus, called in this work the S-fungus, does not belong to the damping-off group fungi, because it:

1) does not kill the seedlings before they emerge from the soil;
2) does not decay the seedlings after they emerged from the soil and whose stems are still soft and succulent;
3) does not cause root rot or top killing;
4) does not decay the infected seeds in seed bed (in four and a half years.

The S-fungus in favourable conditions is capable of destroying

100 percent of the sown conifer seeds.

Control of the S-fungus is possible through pelleting the seeds with Captan.

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* Originals not seen.

More or less circular areas free of seedlings in conifer seed beds.

FIG. 3. Red pine seed bed in Midhurst nursery (1962).

FIGS. 4 and 5. White pine seed beds in Midhurst nursery (1963).


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FIG. 6. Scotch pine seed bed, July 1962, sown in the fall of 1961 with pelleted seeds (normal germination).

FIG. 7. Scotch pine seed bed, July 1962, sown in the fall of 1961 with non-pelleted seeds (no germination).

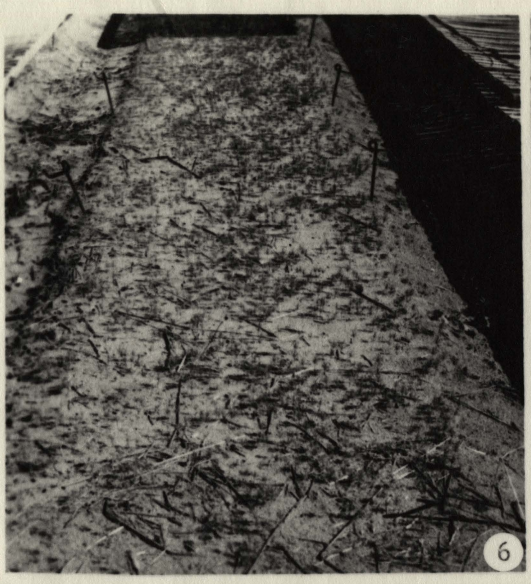
FIG. 8-I Non-germinated red pine seeds from seed beds.

FIG. 8-II Healthy red pine seeds from seed extraction station.

FIG. 9. Outgrowth of S-fungus from a non-germinated red pine seed.

FIG. 10. S-fungus colonies around non-germinated red pine seeds; some colonies have bacterial contamination (peptone dextrose agar, under belljar at 18°C).

FIG. 11. Three month old S-fungus colony on peptone dextrose agar, under belljar at 18°C.



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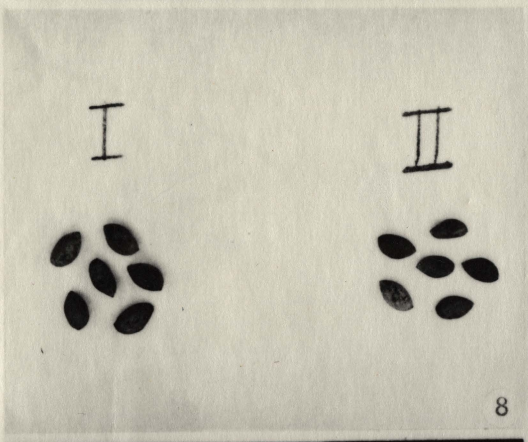

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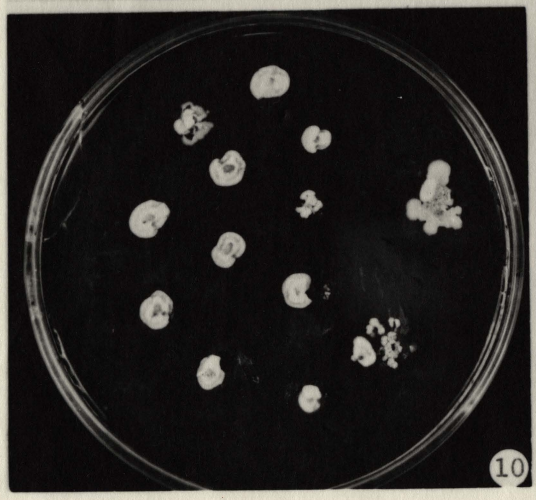

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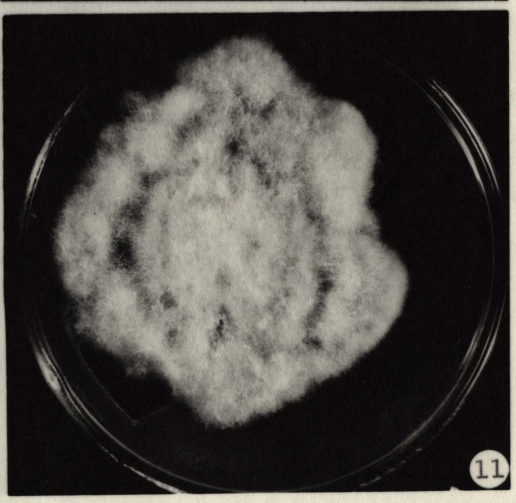

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FIG. 12. Healthy three month old red pine seedlings growing on quartz sand where other seeds were killed by S-fungus (X 1½).

FIG. 13. Red pine seeds placed in quartz sand and inoculated with S-fungus. At 4°C all the seeds were killed within two months (X ½).

FIG. 14. S-fungus sporulation over quartz sand at 18°C after one month of growth (X ½).

FIG. 15. Red pine seed covered with S-fungus hyphae (laboratory inoculation). Two white spots seen on upper margin are compact masses of hyphae (X 12).

FIG. 16. Red pine seeds in quartz sand overgrown with S-fungus hyphae (X 3).


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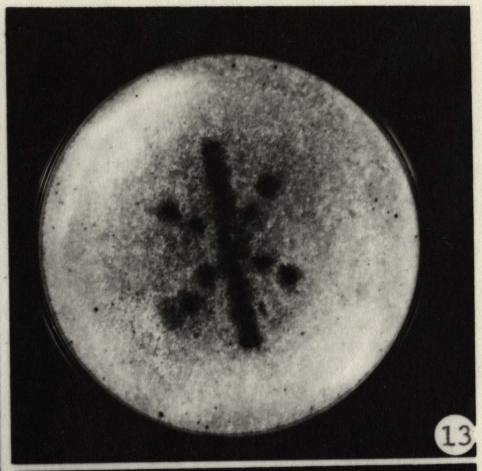
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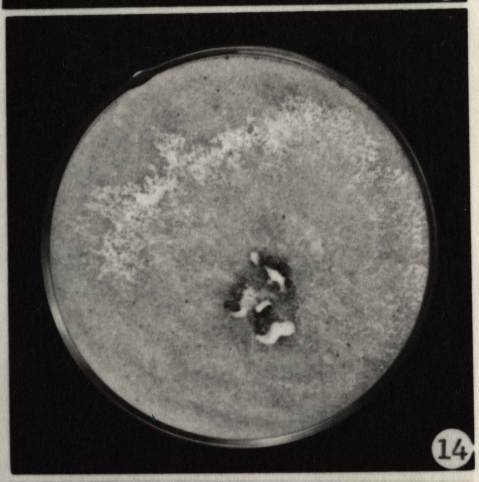
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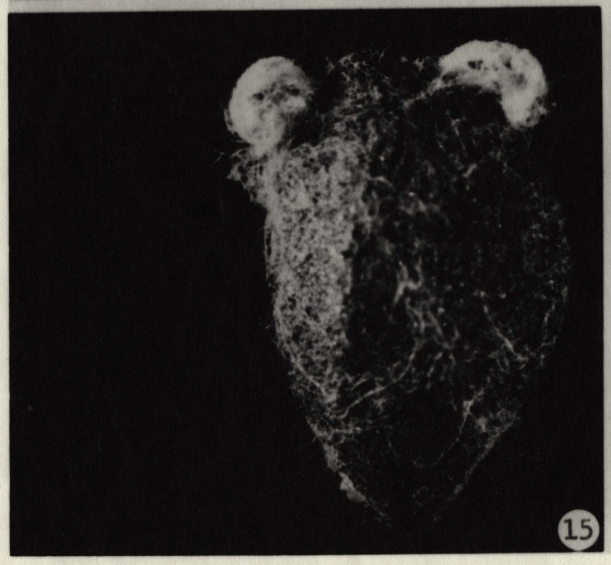
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Growth of the S-fungus on different culture media.

The colonies are six weeks old.

FIG. 17. Peptone dextrose agar.

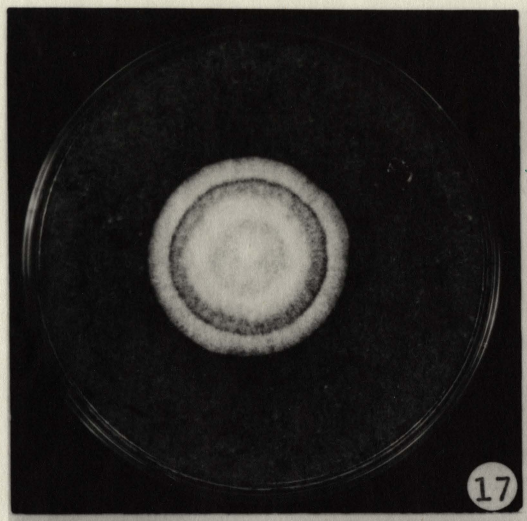
FIG. 18. Corn meal agar.

FIG. 19. Malt extract agar.

FIG. 20. Czapek's agar with Nicotinamide (1 to 1000) but lacking sugar.

FIG. 21. Czapek's agar plus 10g/l yeast extract.

FIG. 22. Czapek's agar without sugar.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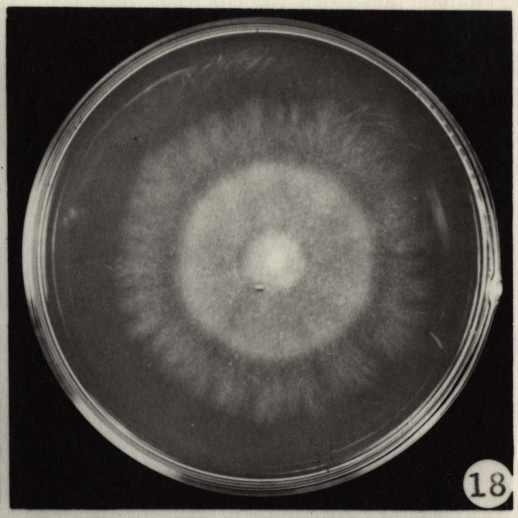
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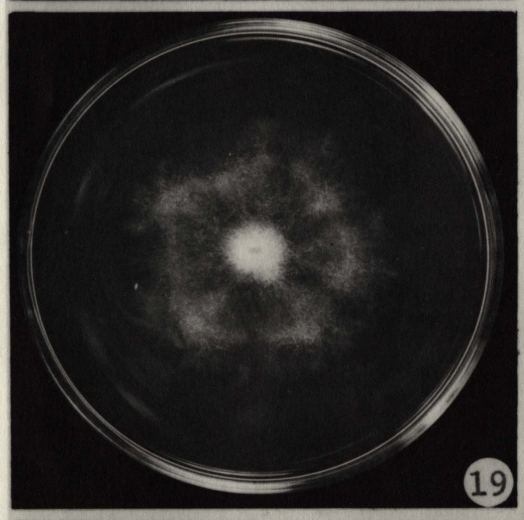
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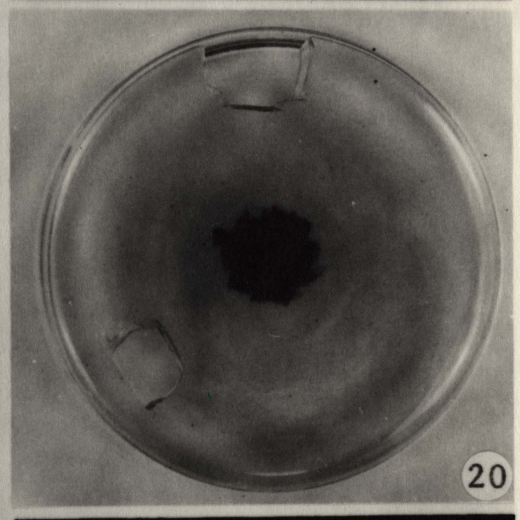
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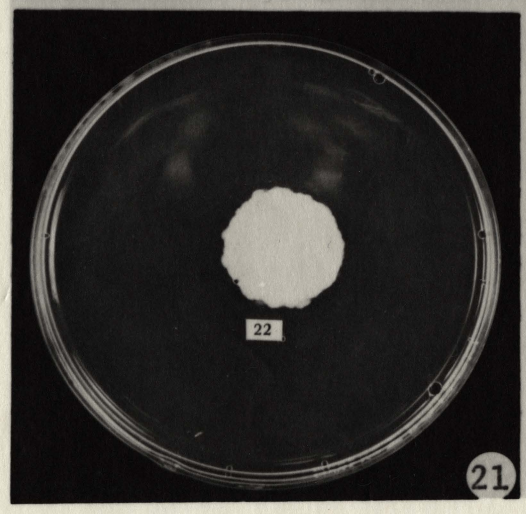
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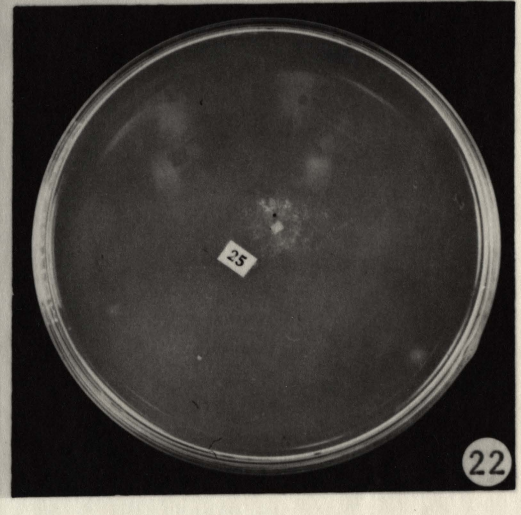
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The influence of different anino acids as extra nutrient sources on growth of S-fungus (1g/l).

FIG. 23. L Alanine.

FIG. 24. beta Alanine.

FIG. 25. Glutamine.

FIG. 27. DL Threonine.

FIG. 28. L (-) Arginine Monohydrochloride.


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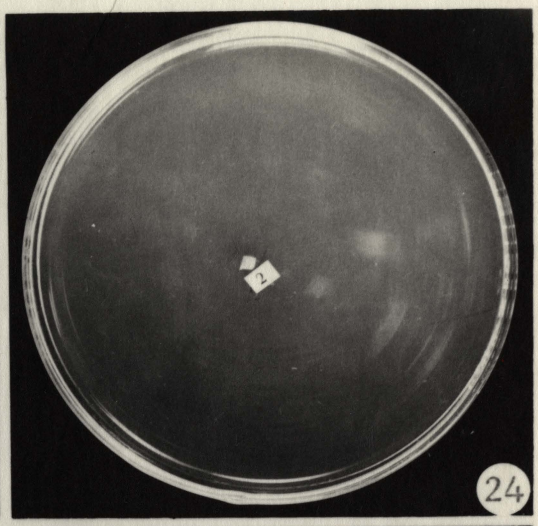
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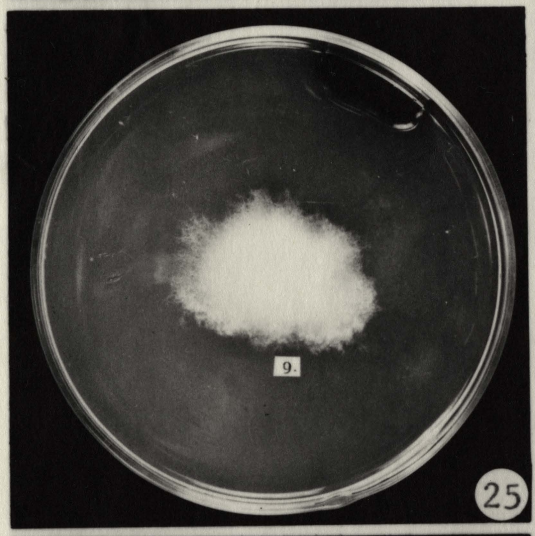
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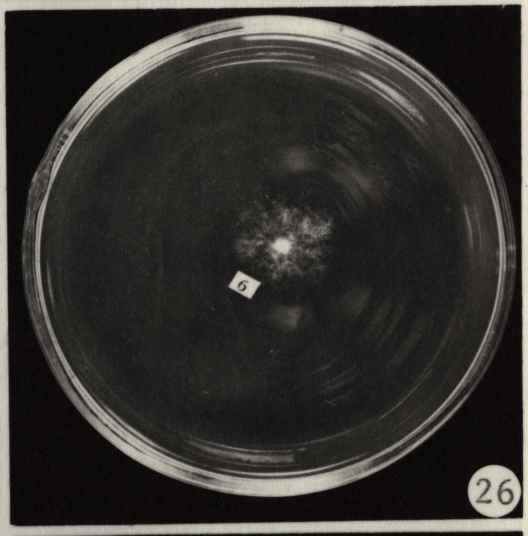
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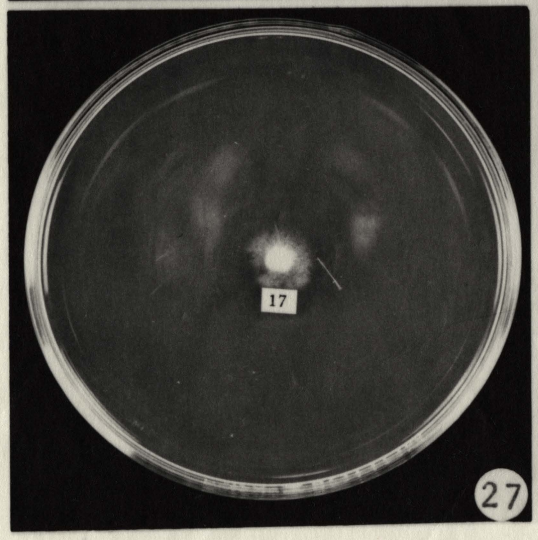
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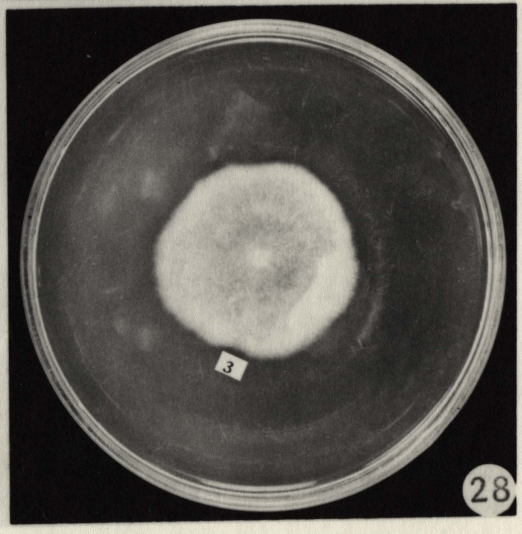
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The influence of different amino acids as extra nutrient sources on growth of S-fungus (1g/1).

FIG. 29. L Aspartic acid.

FIG. 30. DL Glutamic acid.

FIG. 31. DL Phenylalanine.

FIG. 32. DL Tryptophane (no growth).

FIG. 33. DL Histidine HCl (Dihyd).

FIG. 34. L (-) Proline.

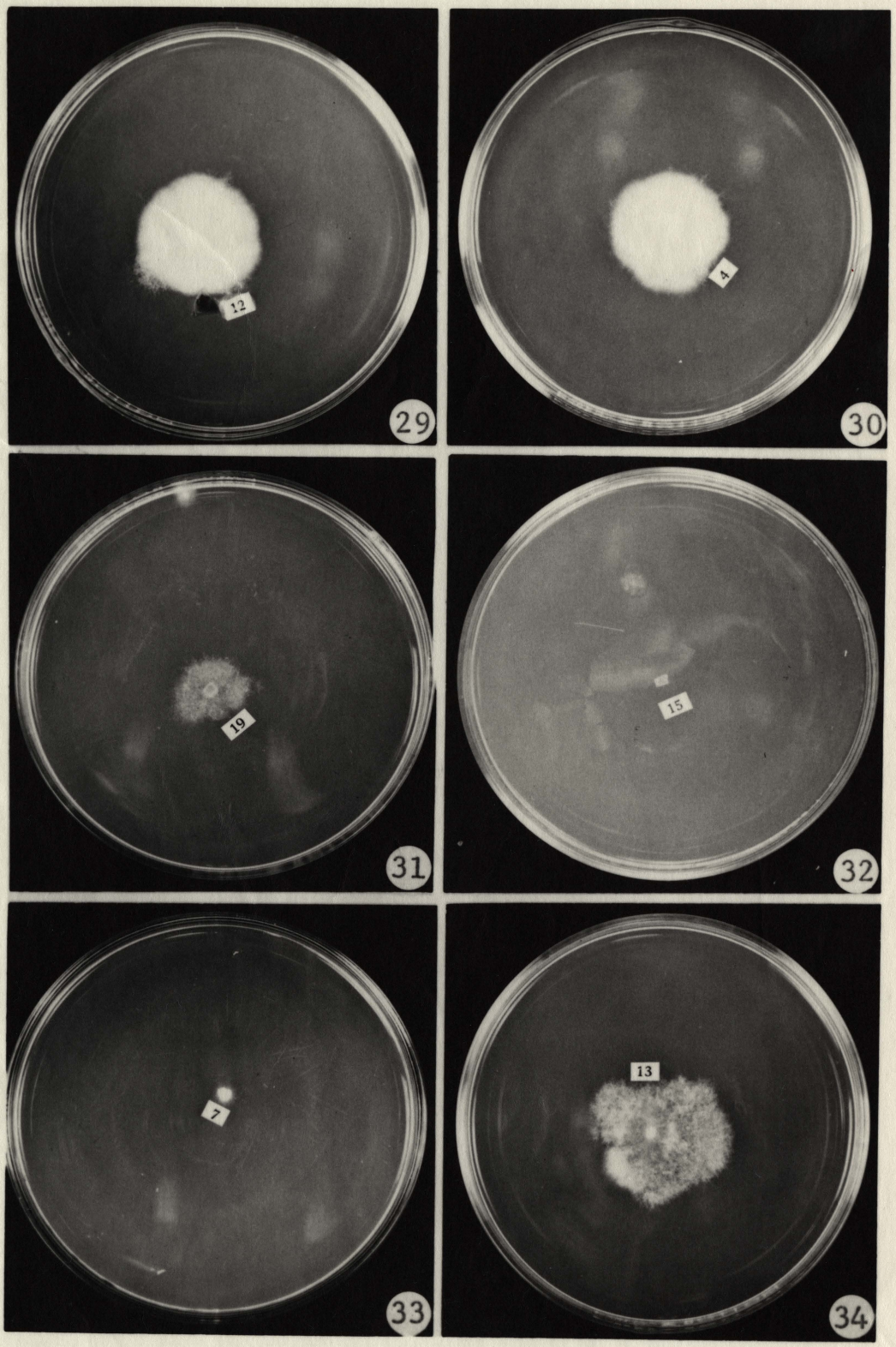
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FIG. 35. S-fungus growth over natural substrata. From left: white pine needles, white pine branches, red pine needles (two tubes) and re red pine branches (X ½).

FIG. 36 and 38. Coloured S-fungus cells from water agar culture at 1^{o} to 3^{o}C (X 750).

FIG. 37. S-fungus colony from liquid Czapek's medium - without sugar, three weeks old - at 15^{o}C temperature (X 7).


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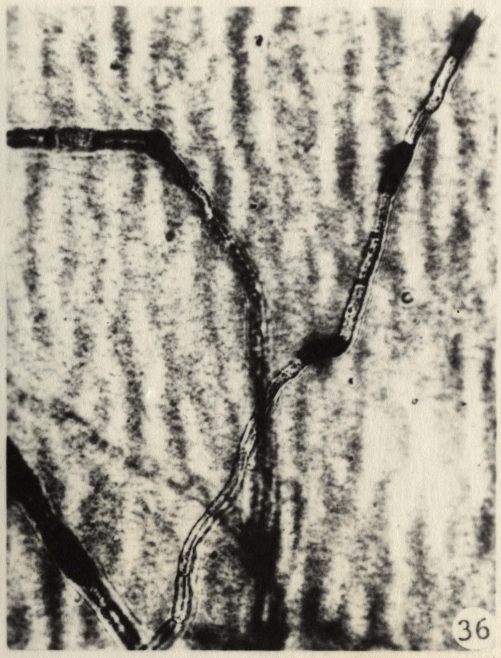
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FIG. 39. S-fungus colony growing over Millipore membrane (X 4).

FIG. 40. S-fungus sporulation over quartz sand (X 50).

FIG. 41. S-fungus hyphae in the seed endosperm (left) and embryo (right) (X 150).


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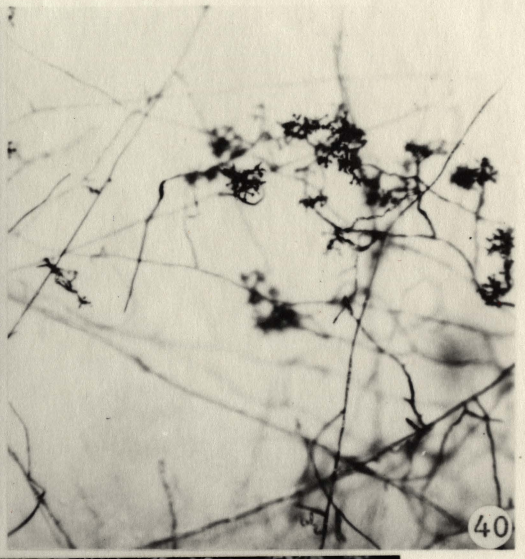
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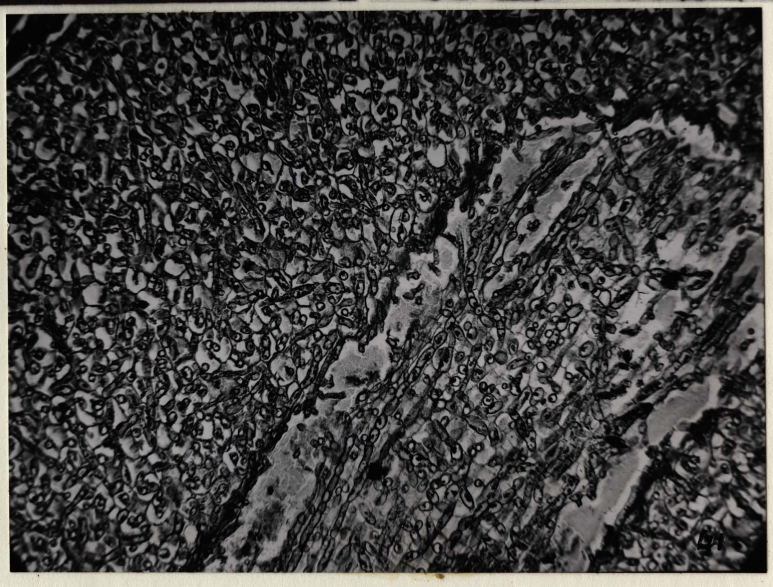

FIG. 42. Pseudoparenchymatic cells from S-fungus mycelium grown on artificially infected red pine seed (X 1500).

FIG. 43-44. Branching and cross connections of S-fungus hyphae (X 1500).

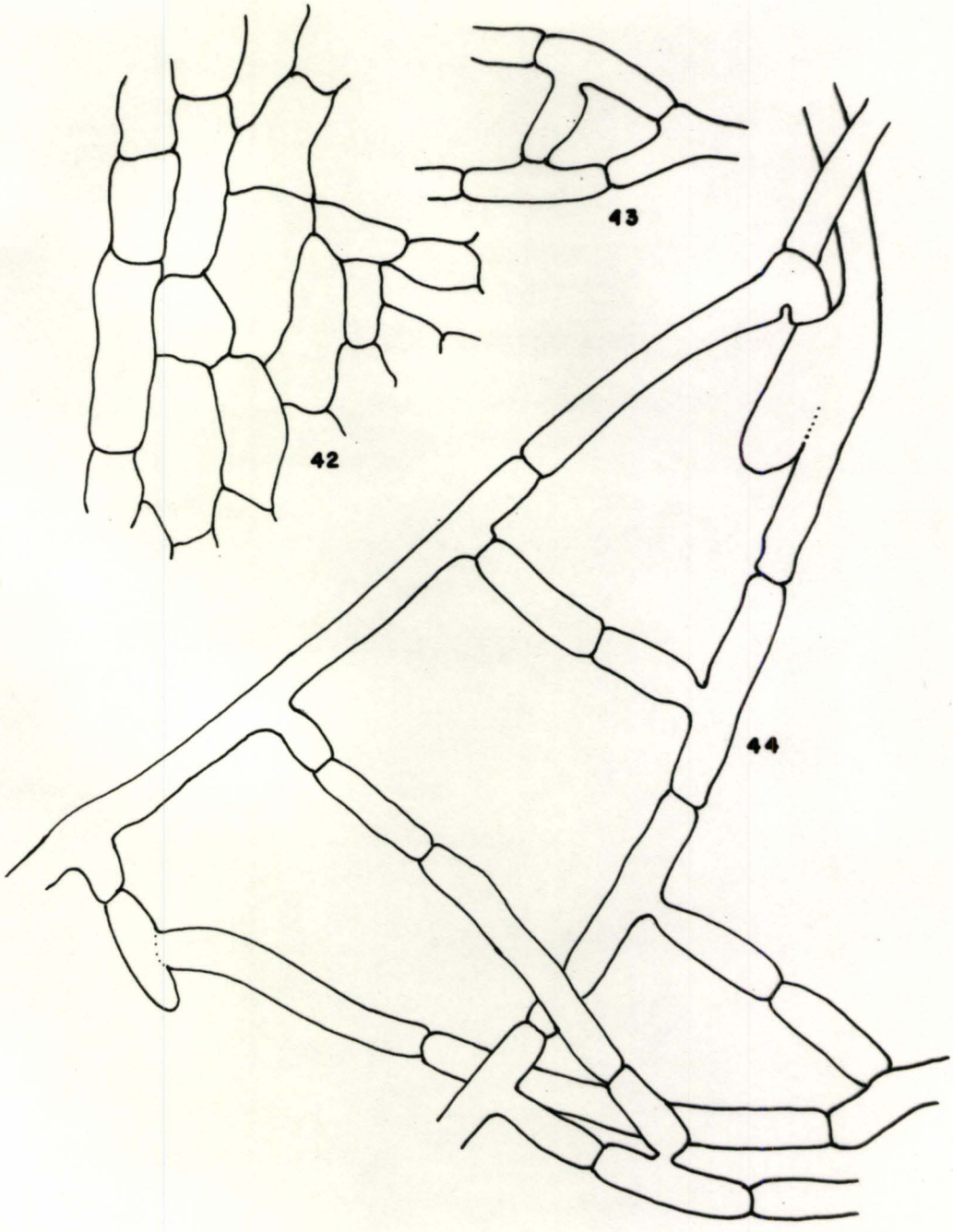
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FIG. 45-52. Development of S-fungus spores (X 1500).

FIG. 53. Matured S-fungus spore (X 3000).

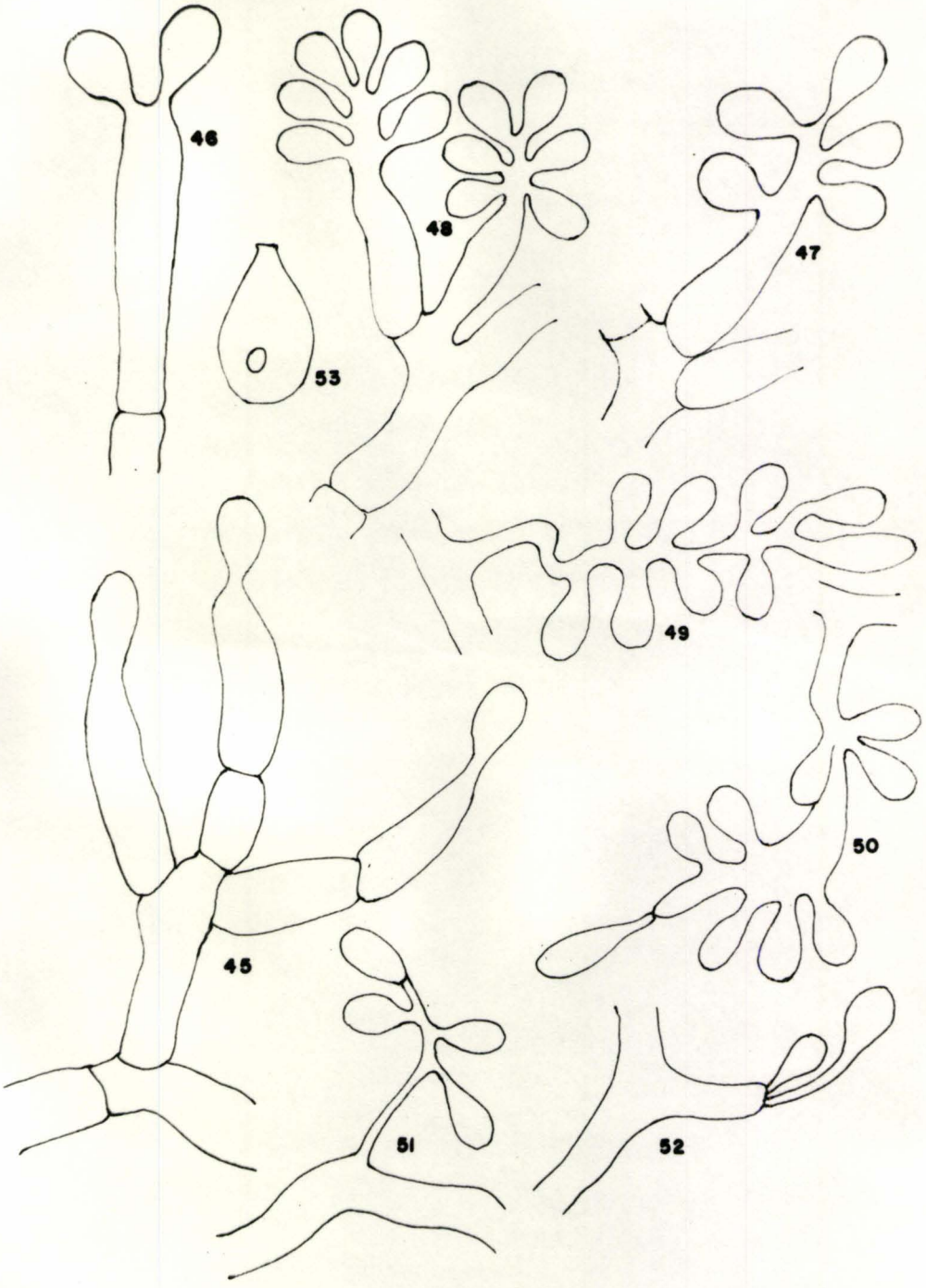
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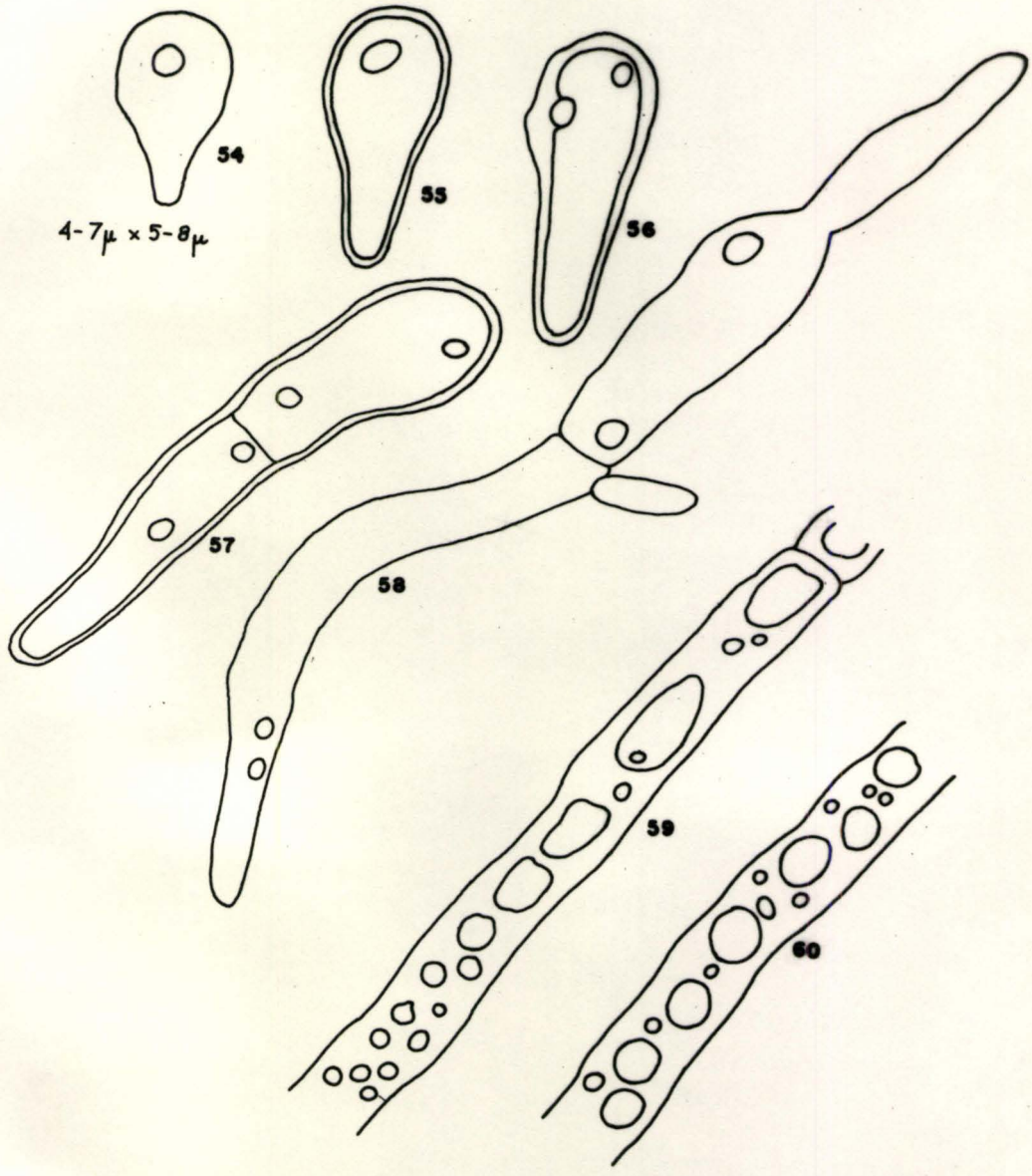
FIG. 54-58. Different phases in the germination of S-fungus spore (X 3000).

FIG. 59-60. Part of cells from S-fungus hyphae (X 3000).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