Some Studies on a Japanese Apple Canker and its Causal Fungus, *Valsa Mali*.

By

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(With Plates XXVII–XXX)

I. Introduction.

About twenty five diseases of apple-trees caused by the parasitic fungi have already been recorded in our country. Among them *Sclerotinia Mali* Takahashi, *S. malicola* M. Miura and *Valsa Mali* Miyabe et Yamada are considered to be of the most economic importance. On the *Sclerotinia* diseases, there are some investigations already done by a few phytopathologists in our country, but as to the *Valsa* disease there are no reports of satisfactory importance, except those of M. Miura (27, 28). In Hokkaido and the Aomori Prefecture great damage has been caused by this *Valsa* disease. In Kamikawa and Iwamizawa districts in Hokkaido, where perhaps the greatest havoc had taken place, the cultivation of apples has been given up in many orchards. It is of utmost importance, that a thorough investigation on this disease should be undertaken.

In the present paper, the results of the investigation on the subject performed during the last three years in the Phytopathological Laboratory in the Hokkaido Imperial University are presented.

The writer wishes here to acknowledge his indebtedness and to express his sincere thanks to Prof. Dr. K. Miyabe and Prof. Dr. S. Ito for their constant and kind direction, and he is also indebted to Prof. Dr. T. Sakamura, Prof. Dr. T. Hemmi and Assistant Prof. Y. Tochinai for their valuable suggestions.

II. Historical Review and Pathogenicity

Since the introduction of the American apple varieties into Hokkaido and Northern Honshu, a new serious disease, peculiar to Japan has sprung up among apple orchards causing a canker disease on trunks and branches. The infected parts are water-soaked in appearance in early spring, becoming dried up later and forming a sunken discolored lesion,

[Jour. of the College of Agric., Hokkaido Imp. Univ., Sapporo, Vol. XII. Pt. 3. Dec. 1924.]

on which numerous fruiting bodies of a fungus can be found and at last causing the death of the diseased trunks and branches.

In 1903, the causal fungus was first described under the name of *Valsa Mali* by K. Miyabe and G. Yamada (38) In the same year, however, S. Hori (16) stated that the apple canker in the Akita Prefecture is caused by *Bacillus amylovorus* (Burrill) De Toni which causes the blight disease of pear and apple trees in America. Since then, various investigators have expressed diverse views on the subject; some attribute the cause of the apple canker to *Valsa Mali*, while others to *Bacillus amylovorus*. S. Hori (16), E. Uyeda (46) and M. Kazui (19) who are supporting the latter opinion, stated in their reports the appearance of the blackish pustules later on the lesion made by the blight bacteria. On the other hand, in Hokkaido, Y. Takahashi (43) proved that the canker is caused by *Valsa Mali*, and S. Kaneda (18) who undertook the study under the anticipation that the cause might be *Bacillus amylovorus*, could not find any bacteria on the various agar and gelatin media infected with the juice from the diseased parts of apple trees. M. Miura (27) after four years' experiments at the Aomori Agricultural Experiment Station came to the same conclusion as Takahashi and Kaneda.

For three years extending from 1921 to 1923, I have undertaken numerous experiments to clear up this problem. For this purpose, I cut a piece of water-soaked or cankered portion from different varieties of diseased apple trees, and inoculated them on the various agar media. Although I have not been able to isolate the blight bacteria, but in every case the mycelia and pycnidia of *Valsa Mali* have appeared invariably on the cultures.

From the above experiment we can conclude that the apple canker at least in Hokkaido and Aomori districts is caused by *Valsa Mali*, but perhaps the apple canker in some other parts of Japan may be caused by *Bacillus amylovorus*. However that requires further investigation and confirmation.

III. Symptoms of the Disease.

The canker appears on the twigs, branches and main trunks of both young and old apple trees, but very rarely on very young trees. The infected bark shows at first a swollen water-soaked appearance, during the months of April and May in Sapporo. If we press the lesion with the point of a finger, it yields readily to the pressure. The epidermis

can easily be scraped off, exposing discolored tissues beneath. No fluids of any kind ever ooze out from the lesion, which later dries up and becomes sunken.

Generally about a month after infection, the black fruiting bodies of the fungus are formed over the entire cankered area. Often cracks are formed on the bark, especially at the margin between healthy and diseased portions. The usual shape of the canker is elliptical, being longer in the direction of the long axis of the branch, and is normally regular but often irregular on the large branch or trunk. The fruiting pustules are at first covered by the periderm, which becomes lifted up and finally ruptured exposing the blackish stromata of the fungus. When the weather is damp from these pustules ooze out fine curling yellowish spore-horns, which are composed of innumerable pycnospores.

As to the symptoms of the apple canker, I can recognize two types, which T. Hemmi (13) has described in *Valsa japonica* of cherry trees; the one is the cankered type and the other is the girdled type which does not form a canker. About the cankered type, I have already stated above. The girdled type appears mostly on the weakened branches or small twigs without presenting a water-soaked appearance, being caused by a rapid growth of the mycelium. If the infected branch or trunk is growing vigorously, the lesions always form the canker, being preceded by water-soaked swollen effused blisters. Sooner or later, however, the canker normally forms the girdling. The rate of its advance depends entirely upon the condition of the tree. In the vigorously growing trees, the advance of the disease is slower than in the case of the trees having less vitality. The part of the branch beyond a girdled region gradually withers and dies. In the vicinity of Sapporo, where the disease is common, it is not rare to find in summer some withered apple branches with brown, shriveled leaves clinging to the limbs. But it is a very rare occurrence, that the newly affected large branch or trunk is girdled and killed in the first year of infection.

IV. Morphology of the Fungus.

a. Stromata.

As already stated, when the infection occurred on a strong growing tree, water-soaked lesion appears at first. Later it shows a cankered symptom, and in the course of time, all over the lesion numerous blackish stromata are thickly produced, making it conspicuous and easy of diagnosis.

In the young stage of the stroma, it is covered by the periderm of the bark. If the stroma in this stage is sectioned, it will be found to consist of a compactly united mass of the light olivaceous hyphae. It gradually increases in size, until it assumes a conical shape under the periderm. From about that time a pycnidial cavity begins to develop and the stroma breaks out, rupturing the periderm. The cavity is at first composed of a simple chamber, which is gradually enlarged assuming an irregularly lobed shape by the pressure of numerous pycnospores which are constantly cut off from the conidiophores and at last fill up the pycnidial cavity.

W. Ruhland (36) has called such a stroma by the name of "Ectostroma" to distinguish it from "Entostroma" in which perithecia are formed. In autumn, an entostroma is formed under an ectostroma or it is independently produced in the diseased area and later in it are produced a certain number of perithecia. As in the case of an ectostroma, an entostroma is also composed at first of densely crowded hyphae, so that in a cross section it appears as a pseudoparenchymatous tissue, in which the host cells are more or less intermixed. Between the ecto- or entostroma and host tissue, we can not detect in our case a "conceptaculum," or a blackish distinct line of demarkation. This is an important character to separate the subgenus *Leucostoma* from the *Euvalsa*, to which the fungus in question belongs.

When ecto- and entostromata are first produced under cork layer, they are light brownish olive in color, but with age the color deepens to olive and finally to blackish olive or brownish green, and lighter in color near the center. When fully matured, they assume usually a flattened conical disk shape.

b. Pycnidia and Pycnospores.

The first outward indicatiom of ectostromata is the appearance of numerous little black pustules breaking out of the periderm of the bark. In each of such pustules a single pycnidium is formed. If we make a section of the young stroma, a light colored portion is seen in its center showing the formation of a single pycnidial cavity.

The development and growth of the pycnidia were followed by the writer on artificial media. The first stages can be watched directly under the microscope on a nutrient agar film in a Van Tieghem cell (see "Germination of Pycnospores"). In summer temperature, after about twenty four hours, the pycnospores are germinating, and in four or five days, the hyphae divide into short, more or less rounded and light brown

colored cells in certain older portions of the mycelium which become entangled thickly. Little blocks of the agar medium in a Petri dish or test tube, in which the stromata had been produced, were fixed, imbedded in paraffin and sectioned to study the later stages of development. In about 10 days after the inoculation the ectostroma is composed of a solid tangle of undifferentiated hyphae. About half a month after, the hyphae become loosened at the center. The hyphal branches which have extended into this loosened area, forming a closely set layer or hymenium, are the conidiophores, on which the pycnospores are formed. The conidiophores are either simple or branched and the pyconospores are freely and successively cut off from their ends, Thus the cavity is naturally filled with the spores, and apparently by the pressure thus produced the enlargement of the cavity is caused.

In the mature stage of the pycnidia both in nature (Pl. II, Fig. 1) and on culture media, the size and shape of their cavities are quite variable, being irregular or labyrinthiform. The cavity communicates to a number of chambers, having a single exit. The pycnidia in nature measure 0.48 – 1.60 mm. in diameter and 0.40 – 0.96 mm. in height. Only one pycnidium is formed in one stroma, and its wall is not clearly distinguishable from the surrounding tissue of the stroma. From every point of the inner wall of the pycnidium, conidiophores are densely produced. The conidiophores are (Pl. I, Fig. 2) simple or branched and have unequal lengths, the majority being 10.5 – 20.5 μ long and 1.2 – 2.0 μ in diameter. The length of the conidiophores on culture media is much longer than that formed in nature. From the tips of the conidiophores the spores are cut off in succession, so that the cavity is soon filled with them. When moisture is absorbed the mucilaginous substance enclosing the spores is forced out through the ostiole, forming a curling filamentous spore-horn.

The spore-horn is yellowish in color and has a waxy appearance. Under a dried condition it takes a golden yellow color, and it is hard and brittle. But in rainy weather, the horn swells up first and then the mucilaginous substance dissolves away in rain water, washing the spores down the bark. As soon as the rain is over, new spore-horns appear generally with surprising rapidity.

The size of the pycnospores is 4.0 – 10.0 × 0.8 – 1.7 μ (Tables 1 and 2). They are allantoid in shape with rounded ends, colorless and contain a few oil drops (Pl. I, Fig. 4).

c. Perithecia.

According to my observation in Sapporo, the entostroma is produced in late autumn, and remains immatured for a long time. In the first part of February, perithecia reach their maturity in the entostroma which is formed either directly under the ectostroma on an old canker or independently without any relation with the ectostroma. The matured entostroma has on its exposed surface many minute black papillae which project slightly above its surface. Each papilla is an opening of a long neck connected with a perithecium (Pl. II, Fig. 2). A section of an entostroma will show that spherical or subglobose perithecia partly intrude into the host tissues. Their shapes are often variable by the pressure of other perithecia. The number of the perithecia in a stroma are three to fourteen; but commonly four to nine. The matured perithecium measures about 320 – 540 μ in diameter and the length of its neck is variable, ranging from 450 μ to 860 μ. The wall of the matured perithecium is composed of four to six layers of thick walled cells and is about 60 – 30 μ in thickness. The wall of the matured perithecium is thinner than that of the younger one, and cells of the former are much flattened.

d. Asci and Ascospores.

When fully matured, the cavity of the perithecium is closely packed with asci which are formed from the basal inner wall of the perithecium. The asci (Pl. I, Fig. 1) are clavate-oblong or clavate-fusiform, rounded or truncate at the apex and subsessile, measuring 28 – 35 × 7.0 – 10.5 μ (Table 6). The wall of the ascus is hyaline and much thickened at the apex and less so at the base. At the upper extremity of the lumen of the ascus, a pore-like portion is seen and in dried specimens this portion is very prominent. With potassium iodide solution of iodine, the wall of the tip is not colored. There are many oil drops in the cavity of the ascus as well as in the ascospores.

The matured asci always contain eight ascospores and their arrangement is subbiseriate or irregular. The ascospores are simple and allantoid in shape with rounded ends. They are 7.5 – 10.0 × 1.5 – 1.8 μ in size (Table 5), slightly larger than the pycnospores.

e. Mycelium.

The hyphae of the fungus invade the living tissues of the bark, sapwood and even the heart-wood. They are especially conspicuous in the vessels of the vascular bundles, running up and down longitudinally (Pl. II, Fig. 3). If the cut surface of the cross section of the diseased trunk or branch is carefully observed, it will be found that the diseased surface is light brown in color, easily distinguished from the healthy portion which is whitish yellow in color. The diseased discolored portion has an irregular outline and often extends over the heartwood. When the block of the diseased branch is kept in a moist chamber, the white mycelium grows out from the cut surface in a few days. As the hyphae destroy the cambium cells, the diseased branch ceases its growth on the affected portion. As a consequence, the formation of annual rings becomes irregular making cross sections of such a branch to assume various shapes.

The mycelium of the fungus in host tissues varies from 2.0 to 7.5 μ in diameter and is hyaline or slightly colored, but on the culture media it becomes olive or brownish olive in color. The mycelium in the host tissues (Pl. II, Fig. 4) and cultures (Pl. I, Fig. 7) contains many oil drops, and especially so in winter.

V. Measurement of Pyconospores, Asci and Ascospores.

In the measurement of pycnospores, the spores which had oozed out from fresh material under a moist condition were used. On account of the difficulty of overcoming the motion of the spores in a fluid medium, they were mounted in the potassium-glycerine-copper medium, which is recommended by Miss Tiller (39) in the spore measurement of *Endothia parasitica*. The formula is as follows:— 1 part of 2 % aqueous solution of potassium acetate, 1 part of 40 % alcoholic solution of glycerine, and copper acetate sufficient to color. The number of measurements of the pycnospores is one hundred per each variety of diseased apple trees. And the number of the varieties selected is five, making the total number of the pycnospores five hundred.

M. Miura (27, 28) has reported the size of the pycnospores to be 7–10× 1.0–1.5 μ. According to my measurements, their length is 3.0–10.0 μ, and width 0.8–1.7 μ. The maximum of their length and width is more than

twice the minimum. Such variation in the size of the pycnospores, as in the case of asci and ascospores is caused by the grade of their maturation and surrounding conditions. The detailed results of measurements are shown in Tables 1 and 2.

Also, I measured an equal number of pycnospores which had oozed out from pycnidia formed on the media of bark decoction agar, apricot agar, synthetic agar No. 2, and malt agar. The difference in size between the pycnospores which were produced under natural conditions and the bark decoction agar medium, was almost negligible but on four other media they were far smaller than the natural ones in both length and width (Table 3 and 4).

Two hundred measurements were taken for both asci and ascospores produced in nature. As shown in Table 5, the size of the ascospores is somewhat larger than that of pycnospores, although we can scarcely detect their difference under the microscope. When the asci are dissolved leaving the ascospores in the perithecia, as is often found in nature, we can hardly distinguish which is which, judging simply from the size and shape of spores.

The length and width of the asci are very variable as shown in Table 6.

Table 1. Measurement of the Pycnospores. 1.

Length in Micron.

Class / Vars. of Apple	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	Mean	Standard Deviation
Alexander	1	5	21	22	25	12	6	4	2	1	1	0	0	5.890±0.086	±0.855
Smith Cider	0	5	12	14	27	20	13	5	1	1	1	1	0	6.150±0.092	±0.922
Summer Queen	1	3	14	30	30	16	3	1	1	0	0	1	0	5.835±0.117	±1.169
Iwai	0	6	16	28	32	14	1	1	0	0	1	0	1	5.765±0.079	±0.793
Jonathan	1	4	19	31	29	14	1	1	0	0	0	0	0	5.830±0.060	±0.598
Total of Frequency	3	23	82	125	143	76	24	12	4	2	3	2	1	5.856±0.036	±0.816

Table 2. Measurements of the Pycnospores. 2.

Width in Micron.

Class / Vars. of Apple	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	Mean	Standard Deviation
Alexander	0	1	3	5	23	32	21	11	3	1	1.310±0.014	±0.139
Smith Cider	0	2	9	10	15	29	16	15	3	1	1.289±0.0169	±0.169
Summer Queen	0	1	1	1	7	40	39	9	2	0	1.347±0.0103	±0.103
Iwai	0	1	1	2	15	36	33	9	2	1	1.335±0.0117	±0.117
Jonathan	1	1	6	9	33	31	17	2	0	0	1.243±0.0121	±0.121
Total of Frequency	1	6	20	27	93	168	128	46	10	3	1.305±0.061	±0.137

Table 3. Measurements of the Pycnospores on Culture Media. 1.

Length in Micron.

Class / Culture Media	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	Mean	Standard Deviation
Bark Decoction	0	0	0	2	11	22	36	18	7	3	1	5.975±0.085	±0.854
Apricot Juice	0	0	4	17	34	32	10	2	1	0	0	5.185±0.039	±0.388
Syn. No. 2.	0	1	3	17	34	28	12	5	0	0	0	5.205±0.040	±0.404
Malt Decoction	3	22	33	32	8	2	0	0	0	0	0	4.130±0.034	±0.336
Total of Frequency	3	23	40	68	87	84	58	25	8	3	1	5.124±0.043	±0.851

Table 4. Measurements of the Pycnospores on Culture Media. 2.

Width in Micron.

Class / Culture Media	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	Mean	Standard Deviation
Bark Decoction	0	0	0	1	0	19	39	17	15	6	4	1.359±0.0136	±0.136
Apricot Juice	0	0	1	21	16	35	20	5	2	0	0	1.1175±0.0127	±0.127
Syn. No. 2	0	6	13	36	20	17	4	3	0	0	0	1.503±0.0145	±0.145
Malt Decoction	2	17	16	36	12	14	3	0	0	0	0	0.993±0.0141	±0.141
Total of Frequency	2	23	30	94	48	85	66	25	17	6	4	1.130±0.010	±0.206

Table 5. Measurements of the Ascospores.

Length in Micron.

Class	7.0	7.5	8.0	8.5	9.0	9.5	10.0	10.5	11.0	Mean	Standard Deviation
Frequency	2	11	34	54	52	33	9	4	1	8.76±0.0497	±0.703

Width in Micron.

Class	1.4	1.5	1.6	1.7	1.8	1.9	2.0	2.1	Mean	Standard Deviation
Freqnency	2	27	48	87	27	5	3	1	1.671±0.0079	±0.112

Table 6. Measurements of the Asci.

Length in Micron.

Class	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	Mean	Standard Deviatian
Frequency	2	3	3	6	21	24	34	24	23	20	18	11	8	5	2	3	0	1	1	31.505±0.218	±3.089

Width in Micron.

Class	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	10.5	11.0	11.5	12.0
Frequency	1	1	5	15	24	27	30	31	16	16	13	7	5	4

12.5	13.0	13.5	14.0	14.5	15.0	Mean	Standard Deviation
1	1	2	1	1	1	8.946±0.111	±1.581

VI. Determination of Oil Drops in the Spores and Hyphal Cells.

a. Pycnospores.

Even with a microscope of high power, it is with difficulty that we find the contents of the pycnospores of the present fungus, as well as of its ascospores to be not homogeneous. But if we examine them stained with methyl-green, carbol-fuchsin, Delafields' haematoxylin, etc., we may be able to know that the spores contain unstainable globular substances, especially at both ends of the spores.

M. Miura (27, 28) reported that oil drops are not contained in either the pycnospores, or in the ascospores of *Valsa Mali*. As it seems to me somewhat doubtful, I tried to settle the question by making the following experiments.

1. Alkannin solution.

Dissolved alkannin, manufactured by Gehe & Co., sufficiently in absolute alcohol, adding to it the same volume of distilled water, and filtered the solution through filter paper. The spores were soaked in this reagent for about five days under a moist condition, then the globular substances in the spores were stained a deep red color.

2. Sudan III.

I have made this reagent by dissolving 0.01 gr. Sudan III in 5 cc. of alcohol of 94 per cent and added to it 5 cc. of glycerine. With this reagent the small drops in the spores were stained a red color within four days. This reaction was accelerated by warming.

3. Cyanin.

This reagent was prepared by dissolving 0.1 gr. of cyanin in 10 cc. of 94 per cent alcohol, and adding 10 cc. of water; and then mixing one part of this stock solution to three parts of 94 per cent alcohol. The globular substances in the spores were stained a deep sky blue color in ten minutes.

4. Osmic acid.

One per cent water solution of osmic acid stained these globular substances tawny brown in two days.

From the above mentioned staining reactions we may know that the globular substances in the pycnospores are oil-drops (Pl. XXVII, Fig. 4).

On the conidiophores, I have many times repeated the tests with the above mentioned reagents, but the results have always been negative.

b. Asci and Ascospores.

Oil-drops which the asci and ascospores of the present fungus contain, can hardly be seen even under a high powered microscope without staining. If we treat them with iodine solution, etc., we may be able to find that they are full of many small globular substances. When the asci and ascospores were stained with Sudan III, osmic acid, etc. as stated in the case of the pycnospores, the fatty globules are stained in the characteristic color respectively (Pl. XXVII. Fig. 3).

c. Mycelium.

In this case, I have made the microchemical tests on the mycelium, the first on one in the pure culture media (Pl. XXVII, Fig. 7) and the second on the mycelium in the wood tissues of the apple tree (Pl. XXVIII, Fig. 4). In every case, it has been ascertained that there are many oil drops which reflect light strongly. Especially they are very abundant and large sized in the vigorously growing hyphae. However, I could not determine the presence of similar drops in old and colored hyphae of the stromata, etc.

From the above stated reactions, we may with certainity conclude that the globular bodies contained in the cytoplasm of the pycnospore, ascospore, ascus and mycelium of the present fungus are oily substances.

VII. Isolation.

The causal fungus is most easily isolated by removing to an agar medium a part of the spore-horn oozing out from a pycnidium in nature. Generally, it can be found when the weather is moist and sultry, though I have observed fresh spore-horns at the beginning of November, and even in April soon after the snow has melted. However, if a piece of the diseased branch or bark is kept in a moist chamber, a yellowish spore-horn oozes out readily from each stroma and if we sterilize beforehand the exposed surface of the diseased part, we can safely isolate the uncontaminated pathogen from it. Also, the fungus may be isolated by transferring, after sterilization of the exposed surface, a piece of the diseased bark or wood to agar media.

On June 20, 1923, after sterilizing the surface of the lesions on seven apple varieties, Newtown Pippin, White Pippin, Jonathan, Tolmans Sweet, Alexander, McIntosh Red and Wealthy which were affected under natural condition, I cut out a small piece of the bark with a water-soaked appearance, with a sterilized scalpel and transfered each piece to the agar plate of bark decoction, potato, corn meal, bean and apricot agar media. All the plates showed no contamination of bacteria, but only the growth of the mycelium, culture characteristics of which are identical with those of *Valsa Mali* on general culture media inoculated with a piece of the spore-horn, and later on the mycelium are produced the stromata. In 1922, through the kindness of Ass. Prof. Y. Tochinai, I was able to examine the diseased branches of an apple tree which were sent to him from Kuroishi, Aomori Prefecture. The specimens had the lesions with a water-soaked appearance and were more or less shriveled, but no stromata had been produced. With the same method as above mentioned, I removed a small piece of the diseased part to various agar media and succeeded in obtaining pure cultures of *Valsa Mali*. During the period of 1921 to 1923, I have many times repeated such isolation experiments with the water-soaked and cankered lesions of the apple tree, but have always failed to prove the existence of *Bacillus amylovorus*.

From these experiments and from the data of Miura's (27, 28) and Kaneda's (18) experiments, we may safely state that *Bacillus amylovorus* is not connected with the apple canker at least in Hokkaido and in the Aomori Prefecture.

VIII. Inoculation Experiment.

Experiment 1.

This experiment was undertaken in order to determine the parasitism of the present fungus and also the resistant power of different varieties of apple trees to its attack. On August 19, 1921, with or without incision, and burning, the apple varieties which were three years old and which had been planted in the spring of the same year, were inoculated with the pycnospores taken from the spore-horns that oozed out from the sterilized twig.

a. With incision and burning.

In this experiment, for the inoculating purpose I have used sound saplings of 12 apple varieties, *viz.*, McIntosh Red, Jonathan, Tompkins King, Iwai, Alexander, Fameuse, Northern Spy, Ralls Janet, Tolmans Sweet, White Pippin, Yellow Transparent and Wealthy.

The bark of the stem to be inoculated is first sterilized with 0.1 % corrosive sublimate and is then washed over and over again with sterilized water. A cross slit on this portion was made with a scalpel which had been brought to red heat. By burning, the neighbouring tissues of the wound were killed. This operation was based upon the idea that the fungus may first require the dead cells for the penetration of its germ-tubes, as far as possible imitating natural old wounds. The spores were introduced with a cold sterilized needle and the wounds were covered with moist absorbent cotton and then with paraffin paper for five days. After a day or two until the covering was taken off, the cotton was moistened with water once or twice every day.

When the cotton and paraffin paper were removed, the burned part showed discoloration. On the 10th day, the discolored part was outlined with a white coloring material. With the mycelial growth and invasion, the lesion with a water-soaked appearance increased in size, the rate of its progress being shown by the white rings applied every fifteen days, as shown in Pl. XXIX, Figs. 1, 2, 4, 5, and 6. After 25 days, the saplings of McIntosh Red, Jonathan, Tompkins King, Iwai, Alexander, Ralls Janet, Tolmans Sweet, Fameuse (Pl. XXIX, Fig. 4), Northern Spy (Pl. XXIX, Fig. 5), White Pippin (Pl. XXIX, Fig. 6) were girdled. The mycelial

growth in the bark above the upper limit of the girdling was exceedingly rapid, producing no water-soaked symptom, while that below the lower limit was very slow, producing the characteristic water-soaked appearance. McIntosh Red and Tompkins King were most seriously affected, and after 25 days, the parts above the girdling were dried up and the stromata had already been formed on the lesion near the point of infection. Wealthy (Pl. XXIX, Fig. 2), and Yellow Transparent (Pl. XXIX, Fig. 1), however, seemed to be more or less resistant, showing no girdling even three months after inoculation. On them, nevertheless, the characteristic symptoms of the disease, such as a water-soaked appearance, depression and pycnidia formation, though slight, could be observed.

b. Incision only.

In this case, healthy saplings of nine varieties were selected for the experiment, namely Jonathan, Iwai, Alexander, Ralls Janet, Tolmans Sweet, Yellow Transparent, Fameuse, Northern Spy and Wealthy. Besides them three undergrown saplings of McIntosh Red, Tompkins King and White Pippin, which had a few green leaves on their stems, were used as control.

With the same method as above stated, the bark of the sapling to be inoculated was sterilized with corrosive sublimate solution, and a cross slit was incised with a sterilized cold scalpel and into it the pycnospores were inoculated with a sterilized cold needle. The wound was kept moist by covering with cotton and paraffin paper for five days. Even one month later no symptoms of the disease were found on any of the healthy saplings thus inoculated. But the undergrown saplings were all seriously affected by the rapid invasion of the fungus. The lesion with a water-soaked appearance did not appear on any of them.

c. Without incision and burning.

In this experiment three healthy saplings of Tompkins King, Tolmans Sweet and Yellow Transparent were selected. The surface of smooth bark, lenticels, the base of a new growing shoot and the old stem end that had been cut in spring, were smeared with the pycnospores and the saplings were placed under bell-glass to keep them moist for five days. At the end of three weeks it was found that the results of this experiment were negative with the one exception, however, that on the old stem

ends of every variety, infection took place. In this case, also, the downward growth of the mycelium was very slow, compared with its upward growth.

Experiment 2.

This experiment was intended to decide whether the results of Experiment 1, a. are correct or not. On October 3, 1921, the healthy saplings of the same varieties used in Experiment 1, a. were inoculated with a bit of the mycelium from a pure culture. The methods were the same as in Experiment 1, a. Although on each sapling infection resulted, the lesion did not generally present a water-soaked appearance. Owing to the cold weather of this season in Hokkaido, the progress in growth of the lesions was very slow, many of which overwintered without producing stromata. Next spring, the lesion regained its growth.

Experiment 3.

As in the case of the Experiment 1. a, it was the purpose of this experiment to determine the resistant power to this fungus among apple varieties. On June 20, 1923, healthy saplings of forty six apple varieties were inoculated with the heavy suspension of the pycnospores. The inoculation operation were the same as in the Experiment 1, a. The results are given in the following table:—

Table 7. Showing the results of inoculation (after 2 months).

Jonathan	II	II		Mita No. 54	O	O	I	I
Iwai	II	I		Peasgood Nonsuch	III	O		
Tolmans Sweet	II	I		Akin	I	I		
Yellow Transparent	I	II	II	Ben's Red	III	I		
Fameuse	I	I		Newtone	O	O	O	
McIntosh Red	II			Goryo-beninanako	I	I	I	
White Pippin	I			Yokohama No. 5	I	I		
Wealthy	I	I		Stark Florence	O	O	O	
Ben Davis	I			Cox's Orange Pippin		I		
Gravenstein	I	I		Champion	III	I		
Smith Cider	II	II		King David	I	I		
Red Astrachan	I			Swaar	II	I	I	

Roter Trierer	O			Sudley	I	O		
Peach	O			Pomona	I	O		
Allington Pippin	O			York Imperial	I	O	I	
Ko-murasaki	I			Giant Geniton	I	II		
Beauty of Bath	O			Bethel the Great	II	I		
Winter Pearmain	II			Stayman's Winesap	II			
Shin-yuniu No. 26	I	III		Kure-nishiki	/	O		
Fukoku No. 1	I	I		Delicious	II	O	I	
Esopus Spitzenburg	II	I	I	Schöner von Boskoop	III	II	I	
Skinners Seedling	O	I		Gladstone	O			
Kent	II	O	I	Shin No. 7	II	I	I	I

III shows died. II shows died above inoculated portion. I shows slightly affected.
O shows not infected. / shows died by other injuries.

As seen from Table 7, three saplings each of Stark Florence and Newtone gave negative results, recovering from the wound which was made at the time of inoculation. According to a kind letter of Mr. Z. Shima, Expert of the Aomori Agricultural Experiment Station, Stark Florence, which is a variety of crab-apple, is a very sturdy and vigorous grower, with a small and somewhat astrigent fruit. In our country, it has no economic value. Newtone is also a sturdy grower and has no economic importance. If we succeed in raising hybrids between these resistant varieties and the improved susceptible varieties of common apples, resistant apple varieties with desirable qualities may be obtained.

From the above three experiments we may safely infer the following conclusions:—

1. *Valsa Mali* is the direct cause of our apple canker, and the fungus is a wound parasite requiring old wounds or dead barks caused by winter injury, wind injury, pruning, insects, etc. As T. Hemmi (13) stated about the invasion process in the case of *Valsa japonica*, the germ tube or single hypha of our fungus independently possesses no power to penetrate the living cells of the host. But it thrives in an old wound where the surrounding cells are dead, and produces there a mass of hyphae, which penetrate the living cells by secreting enzymes.

2. If the infection has taken place on the branches or trunks of strong growing apple trees, the infected bark at first presents a swollen water-soaked appearance, then the lesion dries up and the stromata are produced. However, if the infection has taken place in late autumn on normal healthy apple trees when they have already ceased growing, or

on the barks of undergrown weak trees or on the parts above the girdling, the lesion does not exhibit a swollen water-soaked appearance, but assumes at once a sunken canker symptom.

3. According to the author's field observations and inoculation experiments as well, the causal fungus may hybernate with pycnidia or even with mycelia without entostroma.

4. Three saplings each of Stark Florence and Newtone were not affected by this fungus showing that they are almost immune to the canker. But from the standpoint of economic value, these varieties are not sufficiently important to be generally cultivated. However, it is possible that an excellent apple resistant or immune to this fungus may be raised from these varieties.

Experiment 4.

It was undertaken to ascertain whether our fungus can infect other trees which are the host plants of *Valsa ambiens* (Pers.) Fr., *V. leucostoma* (Pers.) Fr., *V. japonica* Miyabe et Hemmi, *V. Paulowniæ* Miyabe et Hemmi, etc.

On September 13, 1921, using the same inoculation method as in Experiment 1. a. nine species of trees belonging to eight genera in six families, were inoculated with a bit of mycelium from a pure culture. Besides them two apple trees were used as control. Out of this number four species, *Quercus glandulifera* Bl. *Pirus communis* L., *Acer pictum* Thunb. var. *Mono* Pax. and *Paulownia tomentosa* Bail. were not infected by the inoculum, showing only discolored bark caused by burning at the time of inoculation. But on the other species, *Populus nigra* L. var. *italica* Duroi, *Salix sachalinensis* Fr. Schm., *Prunus serrulata* Lindl. var *sachalinensis* Makino, *P. yedoensis* Matsum. and *Malus Zumi* Koiz., the experiment showed a positive result. I shall particularly describe each case in detail.

a. *Populus nigra* var. *italica.*

Inoculated trees: one trunk, about four years old; two branches two or three years old.

On about the 20th day after inoculation, the water-soaked lesions appeared on the portions surrounding the points of inoculation and then the parts were sunken. The growth of lesions continued till the middle of November. In the middle of October, the stromata were found on the

sunken areas (Pl. XXX, Fig. 2), and on November 4, the yellowish spore-horns oozed out from every stromata produced on the cankered area. However in the middle of June of the next year, it was noticed that the formation of a conspicuous callus had taken place checking the growth of the canker, showing a complete recovery from the disease (Pl. XXX, Fig. 6). The measurements of the pycnospores are shown in Table 8.

b. *Salix sachalinensis.*

Inoculated trees: two places on a trunk six years old; one twig, two years old.

In this case the advance of lesions was most rapid. On the 20th day after inoculation the length of the lesions measured 30 cm. On the lateral margins of the lesion two long longitudinal cracks appeared. The bark between these cracks assumed a water-soaked appearance, turning yellowish olive in color, which later changed into a blackish sunken lesion. Though numerous stromata were produced in the tissue of the lesion, they did not break out of the periderm in that year (Pl. XXX, Fig. 1). In the next spring the portion above the cankered lesion as well as the lesion itself dried up, stopping entirely the advance of the disease.

c. *Prunus serrulata* var. *sachalinensis.*

Inoculated trees: two places on a trunk, seven or six years old; one branch, five or four years old.

With the growth of the mycelium, the lesion did not take a water-soaked appearance as usual, but became sunken, and here and there gummosis had taken place (Pl. XXX, Fig. 4). About one month after the inoculation pycnidia were produced. In the middle of June of the next year, the trees recovered from the disease, forming a healthy callus.

d. *Prunus yedoensis.*

Inoculated trees: one trunk, twelve or thirteen years old; two branches, three or four years old.

The symptoms were the same as in the case of *Prunus serrulata* var. *sachalinensis*, but gummosis was not produced (Pl. XXX, Fig. 3). By the middle of June of the next year, a healthy callus was formed surrounding the canker (Pl. XXX, Fig. 7).

e. *Malus Zumi.*

Inoculated trees: one trunk, twelve or thirteen years old; two branches, three or four years old.

The characteristic symptoms of this disease, a water-soaked appearance, depression and stromata production resulted (Pl. XXX, Fig. 5) as in the case of *Malus communis.*

Table 8. Measurements of the Pycnospores on Other Infected Trees.

Length in Micron.

Class / Tree	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	Mean	Standard Deviation
Populus nigra var. italica	1	12	30	31	17	3	2	1	2	1	0	5.541±0.099	±0.985
Prunus serrulata var. sachalinensis	1	5	19	35	26	10	3	1	0	0	0	5.635±0.061	±0.612
Prunus yedoensis	0	3	12	29	34	16	4	1	0	0	1	5.855±0.066	±0.657

Width in Micron.

Class / Tree	1.0	1.1	1.2	1.3	1.4	1.5	1.6	Mean	Standard Deviation
Populus nigra var. italica	3	4	53	34	4	2	0	1.238±0.0083	±0.0834
Prunus serrulata var. sachalinensis	2	2	51	31	11	3	0	1.256±0.0090	±0.0898
Prunus yedoensis	3	4	34	33	17	8	1	1.285±0.0111	±0.1108

Experiment 5.

On July 23, 1922, the same experiment as Experiment 3 was repeated with pycnospores which had oozed out under natural conditions. Contrary to the positive results of the inoculation with a bit of mycelium, the results of this experiment proved to be negative.

From the above two experiments we may infer that under favorable conditions, the present fungus has the power to infect the other trees besides the apple tree. Inoculation with the pycnospores always gave a negative result, while it was positive when tried with a bit of the mycelium. In the following spring, however, the affected trees invariably recovered from the disease. These results tell us that the pathogen will not generally affect trees other than the apple in nature.

IX. Germination of the Spores.

a. Pycnospores.

Two methods of artificial germination were used. In the first method, as in the normal drop culture, a drop of the bark decoction or other nutrient solution containing a suspension of the spores, is placed on the center of a cover glass, which is inverted over a Van Tieghem cell. In the second method, a drop of water with the spores in suspension is spread on a cover glass and permitted to dry up at ordinary room temperature. Then, it is covered with a film of a nutrient agar medium and inverted over a Van Tieghem cell. The last method is more desirable for a study of the germinating process of such a minute spore and offers the advantage of keeping the spores stationary.

The time required for the germination of the pycnospores varies widely with the temperature and the medium used. In a drop of redistilled water, rain water or tap water, germination did not take place, although it was carefully examined over and over again. At room temperature in summer, 23°–28° C., the pycnospores in the solution or agar film of the apple decoction required from twenty-four to thirty hours for their germination and at temperature of 10°–15° C., the germination occurred from the third to the fourth day after sowing. From the data given above, it may be suggested that the temperature of the summer period is most favorable for the infection of the pycnospores.

The germination of the pycnospores is preceded by an enormous swelling of the cells, especially in their width (Pl. XXVII, Fig. 8). As already stated the matured pycnospores measure 4.5–7.0 × 1.0–1.5 μ, but the spores just before the production of the germ tubes are 8.5–14.0 × 4.5–9.0 μ in size, and the spores become elliptical, subglobular, cylindrical or ovoid in shape. A few spores divide into two cells (Pl. XXVII, Fig. 6),

as T. Hemmi (13) has noticed in the germinating process of the pycno- and ascospores of *Valsa japonica*. P. J. Anderson (3, 4) who found the phenomenon of the swelling in the pycno- and ascospores of *Endothia parasitica* states as follows: "The swelling of the spores is due not merely to a mechanical imbibition of water; it is really a process of growth. To be sure, dead spores will swell, but only to about half the size attained by living spores."

Usually a germ-tube is pushed out from one end, and later another one starts at the other end of the spore, but rarely the third one also. The germ-tube is at first hyaline, about 2.8–4.0 μ in width, and occasionally swollen at the end to a slight degree. Later, the branching and septation of the hyphae grown out of the germ-tubes take place and the basal or older parts of the hyphae turn into olive or dirty yellow color, entangling in a mass. The mass is said by many authors to be the young stage in the development of a pycnidium. The above stated process is shown by a series of camera lucida drawings of a single spore at short intervals in Pl. XXVII, Fig. 8.

The germinating spores and also the germ-tubes contain many oil drops as in the hyphae (Pl. XXVII, Fig. 6).

b. Ascospores.

In this case, the same methods were followed as described in treating the germination of the pycnospores. The time required to germinate is much shorter than in the pycnospores. At 25° C. in an incubator, the germination occured within fifteen hours after sowing, and the septation and branching of germ-tubes took place at the end of twenty four hours. Even in pure water, the germ tubes pushed out but at last ceased their growth for want of nutrient.

Like the pycnospores, the ascospores swell up before germination, but not to such an extent. The matured ascospores are approximately 7.0–10.0 × 1.5–1.8 μ in size, and just before germination, the spores measure 8.0–15.0 × 6.0–9.5 μ, becoming ellipsoidal or globular in shape. This character differs from that of *Valsa japonica*, in which T. Hemmi (13) observed the ascospores always swell uniformly, keeping their original allantoid form, but agrees with that of *Valsa leucostoma* observed by R. Aderhold (1).

As in the case of the pycnospores, the ascospores before germination are often divided into two cells (Pl. XXVII, Fig. 9). The two-celled

ascospores show higher percentages in number than the similar pycnospores. The division into two cells before germination both in ascospores and pycnospores has already been observed by O. Brefeld (5) on *Valsa ceratophora*, and also by T. Hemmi (13) on *Valsa japonica*.

The other characters in ascospore germination (Pl. XXVII, Fig. 10) are the same as in the case of pycnospores.

X. General Cultural Studies of the Causal Fungus.

The causal fungus was grown on various culture media under room temperature. On August 24, 1921, the spore-horns, which had been produced on sterilized apple twigs, were inoculated on the cultural media in test tubes. According to observations covering one year, soy agar medium (in which mycelial growth is most vigorous) and synthetic solution agar medium No. 5 did not produce stromata at all, while the other media used in the experiment produced them to different degrees. On the cultural media of the bark decoction, malt, apricot and synthetic solution No. 2 and on sterilized twigs were produced well developed pycnidia, from which the spore-masses oozed out, when they had been placed under a proper condition. The most vigorous growth of both creeping and aerial mycelia and abundant formation of pycnidia took place on the apricot agar medium and sterilized apple twigs.

The results of this experiment are given in detail in the following pages.

1. Bark decoction agar slant.

On this medium, the development of mycelium is not vigorous enough to cover the whole surface of the substratum. The mycelium is apt to creep on the medium and the aerial mycelium is scanty. Only on the portion of inoculation does it appear radiately. After ten days the small bunches of hyphae have appeared mainly on the inoculated parts and then they turned into compact stromata, from which the pycnospores oozed out in maize-yellow colored (Ridg. Pl. IV) drops. One month and a half after, the medium assumed a dark livid brown color (Ridg. Pl. XXXIX).

2. Corn meal agar slant.

The medium was prepared by cooking 15 grams of corn meal in

500 cc. of distilled water for one hour at 60°–70° C. Strain through filter paper, then melt 7.5 grams of agar in this fluid. Filter through cotton.

In the case of this medium, after 20 days, creeping mycelium covered all the surface of the substratum. On the inoculated and the upper portion of the slant, white to avellaneous-colored (Ridg. Pl. XL) aerial mycelium developed. In a few tubes appeared blackish bunches of hyphae, which turned into imperfect stromata, from which no spores were produced even after two weeks. The growth of the fungus caused no darkening of the medium.

3. Ground-rice agar slant.

This medium was prepared in the same way as in the case of corn-meal agar medium. The difference of cultural characters between this medium and the corn-meal agar medium was almost negligible, but generally the mycelial growth on this medium was more vigorous. The days after inoculation the creeping mycelium covered the whole surface of the slant, and after a month the parts forming stromata turned light olive brown in color (Ridg. Pl. XL), but the spores were not produced in the stromata.

4. Malt agar slant.

Boil 50 grams of malt in 500 cc. of water for one hour at 60° – 70° C. Strain through filter paper. Melt 7.5 grams of agar in this solution. Filter through cotton.

On this medium, 20 days after inoculation creeping mycelium covered the whole surface of the slant and produced stromata from which oozed out the maize yellow colored pycnospore masses. After one month and a half the aerial mycelium on the inoculated part as well as on the upper part of the slant turned white to avellaneous in color (Ridg. Pl. XL).

5. Bean agar slant.

Boil 200 grams of Lima beans in 500 cc. of water about 40 minutes. Strain through cloth. Add water to this decoction until it makes 500 cc. Melt 7.5 grams of agar in this nutrient solution. Filter through cotton.

This medium showed a vigorous mycelial growth. Ten days after

inoculation the creeping mycelium covered the whole surface of the slant and the inoculated portion turned yellowish olive in color (Ridg. Pl. XXX) and imperfect stromata had already appeared. After 20 days the creeping mycelium assumed a brownish olive color (Ridg. Pl. XXX) but the aerial mycelium was scanty. Finally the substratum was colored seal brown (Ridg. Pl. XXXIX) and the aerial mycelium pale vinaceous drab (Ridg. Pl. XLV).

6. Potato agar slant.

Sliced 500 grams of peeled potatoes as thin as possible and cooked in 500 cc. of water for one hour at 70°–80°C. Strained through cloth. Added enough water to make 500 cc. Melted 7.5 grams of agar in this decoction. Filter through cotton.

On this medium the growth of both creeping and aerial mycelia was vigorous. Ten days after inoculation the medium showed Dresden brown color (Ridg. Pl. XV) and imperfect stromata had appeared. After one month and a half the medium colored snuff-brown (Ridg. Pl. XXIX).

7. Apricot agar slant.

Seedless apricot (dried) 200 grams steeped in 500 cc. water for 24 hours. Strained through cloth. Melted 20 grams of agar in 500 cc. water. Mixed the two, and enough water added to make 1000 cc. Filter through cotton.

On this medium both aerial and creeping mycelia showed very vigorous growth, covering the whole surface of the medium with a very thick hyphal layer and the aerial mycelium colored brownish olive (Ridg. Pl. XXX). After 20 days barium yellow colored (Ridg. Pl. XV) spore masses oozed out from the stromata, which on the 10th day had already been produced. At about same time, the substratum assumed a snuff-brown (Ridg. Pl. XXIX) to a bister color (Ridg. Pl. XXIX).

8. Soy agar slant after Miyoshi.

This culture medium was first proposed by M. Miyoshi. The formula used in my study is as follows:–

Japanese soy	20 cc.
Cane sugar	5 gr.

Conc. boiled onion juice	25 cc.
Agar	1.5 gr.
Distilled water	50 cc.

This medium showed a most vigorous growth of both aerial and creeping mycelia, covering all the surface of the medium with a very thick and closely entangled layer. But stromata and spores were not produced. Twenty days after, the aerial mycelium turned to avellaneous (Ridg. Pl. XL.) to cinnamon-brown in color (Ridg. Pl. XV). After two months the substratum showed seal brown (Ridg. Pl. XXXIX) to aniline black in color (Ridg. Pl. L).

9. Synthetic solution agar slant No. 1.

Distilled water	1000 cc.
Agar	15 gr.
Glucose	20 gr.
Peptone	10 gr.
Dipotassium phosphate	.25 gr.
Magnesium sulphate	.25 gr.

On this medium the mycelial growth was not vigorous. On the 20th day the creeping mycelium covered the whole surface, and the aerial mycelium was poorly developed on the inoculated part and also on the upper part of the slant. Imperfect stromata were produced in this period. The color of the substratum became sepia (Ridg. Pl. XXIX). Two months after it turned brussels brown (Ridg. Pl. III) to black.

10. Synthetic solution agar slant No. 2.

A synthetic solution agar No. 1 without peptone.

This medium shows mycelial growth better than on the last synthetic solution agar slant. After ten days the creeping mycelium covered the whole surface of the medium, and the surface was colored light dull citrine (Ridg. Pl. XVI). and then dull citrine (Ridg. Pl. XVI). After two months the medium showed from dull citrine to olive citrine in color (Ridg. Pl. XVI), and the matured stromata were produced, oozing out spore masses.

11. Synthetic solution agar slant No. 3.

Distilled water	1000 cc.

Agar	15 gr.
Glucose	10 gr.
Peptone	2 gr.
Ammonium nitrate	1 gr.
Potassium nitrate	.5 gr.
Dipotassium phosphate	.25 gr.
Calcium chloride	.01 gr.

On this medium the development of mycelium is poor. Twenty days after inoculation in only a few cases was the slant surface entirely covered with the mycelium. The medium showed Prout's brown in color (Ridg. Pl. XV). Later the substratum colored light natal brown (Ridg. Pl. XL).

12. Synthetic solution agar slant No. 4.

Distilled water	1000 cc.
Agar	15 gr.
Glucose	20 gr.
Ammonium nitrate	1 gr.
Potassium nitrate	1 gr.
Ammonium sulphate	.25 gr.
Dipotassium phosphate	.25 gr.
Calcium chloride	.01 gr.

After 20 days creeping mycelium covered the whole surface of the slant and the medium showed a light yellowish olive color (Ridg. Pl. XXX) and on the inoculated part a few imperfect stromata were produced.

13. Synthetic solution agar slant No. 5.

Distilled water	1000 cc.
Agar	15 gr.
Cane sugar	10 gr.
Dipotassium phosphate	1 gr.
Magnesium sulphate	.2 gr.

On this medium mycelial growth is very poor. After one month and a half the substratum is light grayish olive in color (Ridg. Pl. XLVI).

14. Sterilized apple twigs.

Cut an apple twig to about four inches long. Put it in a test tube and

sterilize twice for one hour in Koch's steam sterilizer with one day's interval.

On the second day after inoculation white mycelium had already appeared, surrounding the inoculated point. On the fifth day the web-like growth of the mycelium was very vigorous and it covered over the whole surface of the twig and penetrated under the bark as well. After 10 days thick globular masses of white hyphae, where the pycnidia are to develop, were produced in large number. After that, the compact united superficial mycelium turned a light yellowish olive (Ridg. Pl. XXX) to a greenish olive color (Ridg. Pl. XXX). On the 20th day the yellowish pycnospore masses oozed out from the pycnidia.

XI. Effect of Cane-sugar, Glucose, Asparagin and Peptone on the Causal Fungus.

As to the best source of nitrogen and carbon for fungi, the results of many investigators seem to indicate that no generalization can be made, for the fungi differ to a greater or less extent in their requirement of these elements. The best source and the most suitable amount of nitrogen and carbon must be ascertained for each organism.

J. L. Weimer and L. L. Harter (47) in trial experiments with eight species of sweet potato storage fungi found that when ammonium nitrate was substituted for sodium nitrate and glucose for cane sugar in Czapek's nutrient solution, a vigorous growth resulted, and showed that *Fusarium acuminatum*, *Diplodia tubericola* and *Botrytis cinerea* thrive on 30 % glucose medium most vigorously. On the other hand, *Mucor racemosus*, *Sclerotium bataticola* and *Penicillium* sp. produced the highest amount of mycelial dry weight on the medium of 10 % glucose. It is a most interesting fact that all these fungi have the power to germinate and grow in such a solution of high osmotic concentration. G. H. Coons (10) who studied accurately the various factors involved in the growth and the pycnidium formation of *Plenodomus fuscomaculans*, indicated in regard to carbon source that the fungus is able to tolerate very rich food supplies, but pycnidium production in solutions is restricted to M/100 or perhaps M/50 sugar concentration. Generally on M/20 solution of carbohydrates a strong mycelial growth was obtained, accompanied by Oidium-like bodies, but no fructifications were formed except in a few cases. Also he found in the case of nitrogen source that "the availability of any particular nitrogen compound is largely determined by the associated carbon compound. For

instance, peptone which carries available carbon, gave a large number of pycnidia with malic acid but none with maltose. Asparagin, which gives the best growth and the greatest number of pycnidia with maltose, gave no pycnidia with malic acid. Glycerol, which seems on the whole to be a poor carbon source, gave with peptone, strong pycnidium production, but with other nitrogen compounds behaved indifferently." N. Suematsu and K. Kuwatsuka (42) experimenting to find the effect of the various strengths of some carbohydrates :– 0, 0.5, 1.0, 2.0, 3.0 and 5.0, % on *Gloeosporium laeticolor*, have concluded that in both cane sugar and glucose the larger the quantity, the better the mycelial growth. But the formation of reproductive bodies is most abundant on the 1 – 2 % solution. The economic coefficient of starch is less than the above mentioned sugars, however, the increase of its quantity resulted in increased growth. Also, they have shown that peptone is a necessary material in the culture of the fungus, but the addition of more than 2 % leads to bad results. T. Hemmi (14) in his studies on the Japanese *Gloeosporium* came to the conclusion that the optimum concentration of cane sugar is 5 – 8 %, of glucose, 5.7 % and of asparagin, 0.5–0.9 %. Also he found that peptone of 5 % is most suitable for the conidial formation of his fungi and that of 0.5 – 2.0 % gives little difference for the mycelial growth.

In order to determine the effects of cane sugar, glucose, peptone and asparagin on the fungus and to ascertain the correlative relations between cane sugar or glucose as a carbon source and peptone or asparagin as a nitrogen source, in various combination, the three following experiments were carried on.

Experiment 1.

Standard medium

Potassium biphosphate	0.25 gr.
Magnesium sulphate	0.25 gr.
Agar	15.00 gr.
Distilled water	1000.00 cc.

To the standard solution five grams of cane sugar were added when asparagin or peptone was used. If cane sugar or glucose was used, five grams of peptone were added to the solution. Consequently, I had two standard media. Filtered the medium and added to it cane sugar, glucose, peptone and asparagin from 1 to 10 percent each, and then sterilized them in Koch's steam sterilizer, twice with one day's interval.

They were inoculated with a drop of heavily suspended pycnospores.

Table 9. Effects of cane sugar, glucose, peptone and asparagin on the hyphal growth of the causal fungus.

%	Cane sugar	Glucose	Peptone	Asparagin
0	+	+	+	+
1	+++	++++	+++	+++
2	++++	++++	++	++
4	+++++	+++++	+	+
6	+++++	+++++	(+)	+
8	++++++	++++++	−	−
10	++++++	++++++	−	−

Remarks.

1. The more we increase the amounts of both sugars, the stronger is the mycelial growth. But no stromata were produced on any series of sugars.

2. In both cases of peptone and asparagin, 1 % medium is most suitable for the fungus, while amounts larger than 1 % more or less hinder the mycelial growth. On the medium of more than 8 % there is no mycelial growth. On the series of asparagin medium, a few stromata were formed.

Experiment 2.

Standard medium

Potassium biphosphate	0.25 gr.
Magnesium sulphate	0.25 gr.
Agar	15 gr.
Distilled water	1000 cc.

To the standard medium, the amounts required for each percentage of cane sugar, glucose, peptone and asparagin were added, forming the

four groups as follows:- Cane sugar and asparagin, cane sugar and peptone, glucose and asparagin, glucose and peptone, with sixteen combinations in each group. They were inoculated by the same method as in Experiment 1.

On the pure standard medium, the mycelial growth was very poor, forming no stromata, and both mycelium and medium did not color.

a. Cane sugar and asparagin.

Table 10. Effects of cane sugar and asparagin on the causal fungus.

Asparagin \ Cane sugar		0	0.5	1.0	2.0
0	Mycelial growth	+	+++	+++	+++ (+)
	Pycnidial production	−	(+)	−	−
0.5	Mycelial growth	++	++++	++++	+++++
	Pycnidial production	−	+++	++	(+)
1.0	Mycelial growth	+++	++++	+++++	+++++(+)
	Pycnidial growth	−	++	+ (+)	−
2.0	Mycelial growth	++	+++	++++	++++++
	Pycnidial production	−	−	+	−

Remarks.

1. On the cane sugar media without asparagin, the mycelial layer was colored olive green (Ridg. Pl. IV) to olive citrine (Ridg. Pl. XIV) and the media changed to yellowish citrine color (Ridg. Pl. XIV).

2. On the asparagin media without cane sugar, the mycelial layer was not colored, and also the media not changed.

3. Aerial mycelium in every medium colored white to light buff (Ridg. Pl. XV).

4, The media containing cane sugar and asparagin changed to buff yellow (Ridg. Pl. IV) to light orange yellow (Ridg. Pl. III).

5. The increase of cane sugar amount indicates a stronger mycelial growth.

6. The increase of asparagin amount is apt to make aerial mycelium stronger and the 1 % medium of asparagin is most suitable for the fungus.

7. Stromata production is most abundant on the medium of cane sugar 0.5 % and asparagin 0.5 %.

b. Cane sugar and peptone.

Table 11. Effects of cane sugar and peptone on the causal fungus.

Peptone \ Cane sugar		0	0.5	1.0	2.0
0	Mycelial growth	+	++	++	+++
	Pycnidial production	−	−	−	−
0.5	Mycelial growth	++	++++	++++	+++++
	Pycnidial production	−	−	−	−
1.0	Mycelial growth	++	++++	+++++	+++++
	Pycnidial production	−	−	−	−
2.0	Mycelial growth	+ (+)	++	++ (+)	+
	Pycnidial production	−	−	−	−

Remarks.

1. On the media of this group, no stromata were produced, but the mycelial growth was most vigorous, compared with the other groups, and out of sixteen combinations, the media of cane sugar 2 % and peptone 0.5 to 1.0 % showed the best growth.

2. On the peptone media without cane sugar, the color of the media was not changed.

3. Aerial mycelium colored white to avellaneous (Ridg. Pl. XV) or to fawn color (Ridg. Pl. XV) and covered the whole surface of the slant, forming a compact buff.

4. The media except the row without cane sugar turned from bister (Ridg. Pl. XXIX) chestnut brown (Ridg. Pl. XIV) to clove brown color (Ridg. Pl. XV).

c. Glucose and asparagin.

Table 12. Effects of glucose and asparagin on the causal fungus.

Glucose / Asparagin		0	0.5	1.0	2.0
0	Mycelial growth	+	+++	+++ (+)	++++
	Pycnidial production	−	+	+	(+)
0.5	Mycelial growth	++	++++	++++++	+++++++
	Pycnidial production	−	+ (+)	+++++	+++++
1.0	Mycelial growth	+++	++++	+++++	++++++
	Pycnidial production	−	++	++++	+++
2.0	Mycelial growth	++	++++	+++++	++++++
	Pycnidial production	−	++	++	+++

Remarks.

1. The pycnidial production on the media of this group was most abundant and it is far superior in this respect compared with the other groups. The stromata were at first covered with white hyphae, which gradually turned blackish from above downward.

2. On the glucose media without asparagin, the mycelial layer colored natal brown (Ridg. Pl. XV) then to clove brown (Ridg. Pl. XV) and at last to bone brown (Ridg. Pl. XV), while the media turned olive citrine (Ridg. Pl. XVI).

3. Aerial mycelium was white to light brownish drab (Ridg. Pl. XLV) in color and more compact than that of cane sugar and peptone group.

4. Both mycelial growth and stromata formation were best on the medium of glucose 2.0 % and asparagin 0.5 %.

d. Glucose and peptone.

Table 13. Effects of glucose and peptone on the causal fungus.

Peptone \ Glucose		0	0.5	1.0	2.0
0	Mycelial growth	+	+++	++++	++++
	Pycnidial production	−	−	−	−
0.5	Mycelial growth	+++	+++++	+++++	+++++
	Pycnidial production	−	−	−	−
1.0	Mycelial growth	+++	+++++	++++++	++++++
	Pycnidial production	−	−	−	−
2.0	Mycelial growth	++	+++	++++	++
	Pycnidial production	−	−	−	−

Remarks.

The media of this group showed resemblance to those of cane sugar and peptone. The color of the mycelial layer in the former, however, was lighter than that of the latter, and it was also the same in the case of the medium.

General summary of Experiments 1 and 2.

The more the increase of cane sugar or glucose used as a source of carbon at least up to 10 %, the stronger the mycelial growth of this fungus becomes, but the optimum concentration of asparagin or peptone as a nitrogen source lies between 0.5 % and 1.0 %. In the percentages larger than the optimum concentration the mycelial growth is retarded, even though sugars are supplied to a large amount. Asparagin has the power to produce the reproductive bodies but its optimum concentration is between 0.5 % to 1.0 %, and the combination of glucose and aspar-

agin is most advisable for the purpose. On the contrary, peptone stimulates the mycelial growth, when the supplies of carbon are sufficient.

Experiment 3.

For the comparison, I have used two other fungi; *Cytospora chrysosperma* (Pers.) Fr. isolated from *Populus nigra* L. var. *italica* Duroi and *Valsa ambiens* (Pers.) Fr. from *Morus bombycis* Koizumi. These fungi were inoculated on the hard agar media of corn meal, oat meal and apricot juice containing $M/2 - M/2^6$ of cane sugar and glucose, respectively.

a. Corn meal containing cane sugar.

Among these three fungi, *Cytospora chrysosperma* grew most vigorously on each medium and produced pycnidia most abundantly. On the medium of $M/2^3$ cane sugar, it produced perfect pycnidia, from which oozed out the yellowish spore-horns. All media did not color even one month after inoculation. The hyphal layer took dull citrine (Ridg. Pl. XVI) in color, later turning blackish, with whitish aerial mycelium.

In *Valsa Mali*, the hyphal growth is most vigorous on the media containing $M/2^3 - M/2^5$ of cane sugar but the pycnidial production is relatively abundant on the medium of $M/2^6$ cane sugar. The larger the quantity of cane sugar, the deeper the coloring of the media was. After about two weeks, the media changed to reed yellow (Ridg. Pl. XXX), and later to from isabella to buffy olive color (Ridg. Pl. XXX). The control medium did not color.

On the media of higher concentrations of cane sugar, *Valsa ambiens* grew most vigorously and also produced pycnidia most abundantly. About one week after inoculation, the media colored light vinaceous purple-vinaceous purple (Ridg. Pl. XLIV), and later deep brownish drab-dusky drab (Ridg. Pl. XLV).

These results are shown in the Table 14.

Table 14. Results on the media of corn meal containing cane sugar.

Conc. of cane sugar	% due to mol	Valsa Mali		Cytospora chrysosperma		Valsa ambiens	
		Mycelial growth	Pycnidial production	Mycelial growth	Pycnidial production	Mycelial growth	Pycnidial production
M/2	17.11	++	−	++++	+++	+++	++
$M/2^2$	8.55	++	−	++++	+++	+++	++
$M/2^3$	4.28	+++	(+)	++++	++++	++	+
$M/2^4$	2.14	+++	(+)	++++	+++	++	+ or −
$M/2^5$	1.07	+++	(+)	++++	+++	++	− or +
$M/2^6$	0.53	++	+	+++ (+)	++	++	− or +
Control		+	(+)	++	+	++	−

b. Corn meal containing glucose.

In *Valsa Mali*, the media did not color, but hyphal layers showed clove brown to olive brown (Ridg. Pl. XL). Also in *Cytospora chrysosperma*, the media did not color, but the hyphal layers showed Saccardo's olive (Ridg. Pl. XVI) to olivaceous black (2) (Ridg. Pl. XVII), and the stromata were covered by aerial mycelium of whitish or light grayish olive in color (Ridg. Pl. XLVI). *Valsa ambiens* changed the media into light purple drab to purple drab (Ridg. Pl. XLV), and later into deep brownish drab to blackish brown (3) (Ridg. Pl. XLV). Differing from the other fungi, it has the maximum point of hyphal growth and pycnidial production on the media containing higher concentrations of glucose.

These results are shown in Table 15.

c. Oat meal containing cane sugar.

Two weeks after inoculation, *Valsa Mali* colored the media containing higher concentrations of cane sugar primrose yellow (Ridg. Pl. XXX). The hyphal layers were comparatively compact on the media which contain the concentrations higher than $M/2^4$ cane sugar and took brownish olive to light brownish olive (Ridg. Pl. XXX) in color, later turning to blackish.

In *Cytospora chrysosperma*, the hyphal growth and the pycnidial production were generally most excellent compared with the other two. The media were changed into celandine green (Ridg. Pl. XLVII) to blackish brown (Ridg. Pl. XLV) in color, and on those of the concentrations higher than $M/2^4$ cane sugar, the stromata were closely formed aggregated all over the surface.

The cultural characters of *Valsa ambiens* on this medium were the same as those on the corn meal media containing cane sugar.

The results are shown in table 16.

Table 15. Results on the media of corn meal containing glucose.

Conc. of Glucose	% due to mol	Valsa Mali		Cytospora chrysosperma		Valsa ambiens	
		Mycelial growth	Pycnidial production	Mycelial growth	Pycnidial production	Mycelial growth	Pycnidial production
M/2	9.91	+++	(+)	+++ (+)	++++	+++	+++
$M/2^2$	4.95	++++	(+)	+++++	++++	+++	+
$M/2^3$	2.48	++++	+	+++++	++++	++	+
$M/2^4$	1.24	++++	+	++++	++++	++	+
$M/2^5$	0.62	+++	(+)	+++	+++	++	+
Control		+	(+)	++	+	++	+

Table 16. Results on the media of oat meal containing cane sugar.

Conc. of cane sugar	% due to mol	Valsa Mali		Cytospora chrysosperma		Valsa ambiens	
		Mycelial growth	Pycnidial production	Mycelial growth	Pycnidial production	Mycelial growth	Pycnidial production
M/2	17.11	+++++	(+)	++++++	+++++	+++ (+)	++
$M/2^2$	8.55						
$M/2^3$	4.28	+++++	+	+++++++	+++++	++++	+ or −
$M/2^4$	2.14	++++	+	+++++++	+++++	+++	−
$M/2^5$	1.07	+++	+	++++++	++++	+++	− or +
$M/2^6$	0.53	++++	+	++++	+++	+++	++
Control		++	+	++	+	++	+

d. Oat meal containing glucose.

In *Valsa Mali*, the higher the concentration, the better the mycelial growth was, but the pycnidial production was most abundant on the media containing $M/2^2 - M/2^3$ glucose. The hyphal layer colored clove brown to olive brown (Ridg. Pl. XI).

In *Cytospora chrysosperma*, the higher the concentration of glucose, the better the mycelial growth and more the pycnidial production. The stromata were produced closely aggregated all over the hyphal layer with whitish to light grayish olive colored (Ridg Pl. XLVI) aerial mycelium.

It is a very interesting fact that *Valsa ambiens* had two maximum points of the hyphal growth and pycnidial production on the media of the highest and lowest concentrations of glucose. Other characters on these media agreed with those on the corn meal media containing glucose.

The results are shown in Table 17.

e. Apricot juice containing cane sugar.

Valsa Mali showed no distinct differences of cultural characters on apricot media, containing different concentrations of cane sugar. The mycelial growth and pycnidial production were uniformly excellent throughout the series.

Cytospora chrysosperma changed the media into light seal brown (Ridg. Pl. XXXIX), covering the surface with the aerial mycelium of whitish or light ochraceous salmon (Ridg. Pl. XV) color.

Except a few cases, *Valsa ambiens* could not grow on such a high acidic medium as apricot juice, even though much sugar was added.

The results are shown in Table 18.

f. Apricot juice containing glucose.

On the media of high concentrations *Valsa Mali* did not grow, while on the media of concentrations lower than $M/2^3$ it grew very vigorously and the hyphal layer colored mummy brown (Ridg. Pl. XV).

In *Cytospora chrysosperma*, the cultural characters on these media agreed with those on the apricot media containing cane sugar.

Valsa ambiens did not grow at all on any of these media.

The results are shown in Table 19.

Table 17. Results on the media of oat meal containing glucose.

Conc. of Glucose	% due to mol	Valsa Mali		Cytospora chrysosperma		Valsa ambiens	
		Mycelial growth	Pycnidial production	Mycelial growth	Pycnidial production	Mycelial growth	Pycnidial production
M/2	9.91	+++++	(+)	+++++++	+++	++++	++++
$M/2^2$	4.95	+++++	++	+++++++	+++++	++	+ or −
$M/2^3$	2.48	++++	++	++++++	+++++	++	− or (+)
$M/2^3$	1.24	+++	+ (+)	++++	++++	++	+
$M/2^5$	0.62	++	+	+++	++++	+++	+++
Control		++	+	++	+	++	+

Table 18. Results on the media of apricot juice containing cane sugar.

Conc. of cane sugar	% due to mol	Valsa Mali		Cytospora chrysosperma		Valsa ambiens	
		Mycelial growth	Pycnidial production	Mycelial growth	Pycnidial production	Mycelial growth	Pycnidial production
M/2	17.11	+++++++	+++	+++++++	++++	−	−
$M/2^2$	8.55	+++++++	+++	+++++++	++++	− or (+)	− or (+)
$M/2^3$	4.28	+++++++	+++	+++++++	++++	−	−
$M/2^4$	2.14	+++++++	+++	+++++++	++++	− or (+)	− or (+)
$M/2^5$	1.07	+++++++	+++	+++++++	++++	−	−
Control		+++++++	+++	+++++++	++++	−	−

Table 19. Results on the media of apricot juice containing glucose.

Conc. of Glucose	% due to mol	Valsa Mali		Cytospora chrysosperma		Valsa ambiens	
		Mycelial growth	Pycnidial production	Mycelial growth	Pycnidial production	Mycelial growth	Pycnidial production
M/2	9.91	−	−	+++++++	+++++	−	−
$M/2^2$	4.95	− or (+)	−	+++++++	++++	−	−
$M/2^3$	2.48	+++++	+++	+++++++	++++	−	−
$M/2^4$	1.24	+++++	+++	+++++++	++++	−	−
$M/2^5$	0.62	+++++	+++	+++++++	++++	−	−
Control		+++++	+++	+++++++	++++	−	−

Summary of the Experiment.

1. For comparative study, three fungi, *Valsa Mali, V. ambiens* and *Cytospora chrysosperma* were taken. Among them, *Cytospora chrysosperma* grew most vigorously on any cultural medium and produced pycnidia most abundantly. These facts tell us that this fungus has the power to utilize the sugars more than the others.

2. *Valsa Mali* and *Cytospora chrysosperma* had the maximum points of hyphal growth and pycnidial formation on the media of corn meal and oat meal which contain comparatively high concentrations of sugars. *Valsa ambiens* on the media of corn meal had the maximum point of hyphal growth and pycnidial production on the highest concentrations of sugars, while in the case of oat meal media it had two maximum points on the highest and lowest concentrations of sugars.

3. These three fungi showed no differences of cultural characters on apricot media, containing various degrees of sugars. *Valsa ambiens* could not grow on such a high acidic medium as apricot juice, even though sugars were added.

4. In the first parts of the cultural experiment, *Valsa ambiens* changed the media of corn meal and oat meal containing sugars, into a brilliant purple color, which was the character peculiar to this fungus.

5. On any mediun, the three fungi used in the experiment did not produce perithecia even three months after inoculation.

XII. Effect of Tannic Acid on the Causal Fungus.

The fact that tannin is widely distributed throughout the plant kingdom, has long been known, and various authors have discussed on the significance of its presence in living plants, and also the effects of tannin on fungi have been studied by many investigators. From the studies of M. T. Cook (9), G. P. Clinton (6), T. Hemmi (13), and others, we learn that fungus growth is sometimes stimulated by a low concentration of tannin, becoming more luxuriant than that on the medium without tannin, but by the concentrations higher than a certain degree, it is thoroughly retarded. Cook (9) who studied the effects of tannic acid on many species of fungi in artificial cultures, concluded that the majority of the species show the greatest retardation at from 0.1 % to 0.4 % tannin.

T. Hemmi (13) who studied the effects of tannic acid on *Valsa ja-*

ponica and also on *V. Paulowniae* and *V. Mali* for its comparison, concludes that "in the high percentages of tannic acid, *Valsa Mali* shows an enfeebled growth sooner than does *Valsa japonica*, since at 0.8 % it makes comparatively little growth, which corresponds to the condition shown by *Valsa japonica* at about 1.2 to 2.0 %. *Valsa Mali* generally fails to make any growth at above 1.2 %., or only a very poor growth is made in a few tubes up to 2 %. At above 5 % the growth or even the germination of *Valsa Mali* entirely ceases. Generally, the toxicity of tannic acid against *Valsa Paulowniæ* seems to be more conspicuous than against *Valsa Mali.*" From the table of Hemmi's results we may know that cultures of *Valsa japonica* in media containing 0.1, 0.2, 0.4 % of tannic acid show a more vigorous growth than in check cultures of potato-juice-agar without tannic acid. *Valsa Mali* grows most vigorously on the media containing 0.1 and 0.2 % tannic acid. But my two experiments on *Valsa Mali* indicate that on the media containing over M/1000 or 0.035814 % of tannic acid, the crop of dry mycelium is less than that on the medium without tannin. These difference in the results of Hemmi and mine may probably be attributable to the difference of the media used.

Experiment 1.

For the standard medium I have used a solution having the following formula:—

Potassium biphosphate	0.25 gr.
Magnesium sulphate	0.25 gr.
Cane sugar	10 gr.
Peptone	10 gr.
Redistilled water	1000 cc.

In each Erlenmeyer's flask of 200 cc. capacity 50 cc. of the standard nutrient solution were used. To each, tannic acid was added in varying mol. They were twice sterilized in Koch's steam sterilizer for 40 minutes with a day's interval and inoculated with a drop of heavily suspended pycnospores. Then they were placed in the incubator of 25° C.

The results harvested three weeks after sowing are as follows:—

Table 20. The effect of the different concentrations of tannic acid on the causal fungus with cane sugar and peptone.

Concentration of tannic acid in mol	Equivalent in percentage	Average weight (gr.) of mycelia in 3 flasks
0	0	0.2121
M/16000	0.002238	0.2199
M/ 8000	0.004476	0.3445
M/ 4000	0.008953	0.3374
M/ 2000	0.017907	0.3268
M/ 1000	0.035814	0.1998
M/ 500	0.071628	0.1652

Experiment 2.

In 1000 cc. of redistilled water were placed 10 grams of glucose in place of the same amount of cane sugar and 10 grams of asparagin instead of the same amount of peptone, otherwise everything was the same as in the case of Experiment 1.

Table 21. The effect of the different concentrations of tannic acid on the causal fungus with glucose and asparagin.

Concentration of tannic acid in mol	Equivalent in percentage	Average weight (gr.) ef mycelia in 3 flasks
0	0	0.2015
M/32000	0.001119	0.2662
M/16000	0.002238	0.2498
M/ 8000	0.004476	0.2873
M/ 4000	0.008953	0.2591
M/ 2000	0.017907	0.2227
M/ 1000	0.035814	0.1890
M/ 500	0.071628	0.1972
M/ 250	0.143256	0.1123

From the preceding experiments, the following conclusions may be drawn. Tannic acid has a tendency to stimulate or retard the growth of the causal fungus. In both experiments, the growth was stimulated by low concentration of tannic acid. The media containing M/32000, M/16000, M/8000, M/4000 and M/2000 of tannic acid showed more luxuriant my-

celial growth than on the medium without it. As the amount of the tannic acid was increased, it retarded the growth of the fungus, i. e. the media containing M/1000, M/500 and M/250 of tannic acid showed poorer growth than on the control medium.

XIII. Relation between Temperature and Mycelial Growth.

Experiment 1.

The investigations of the relation between temperature and mycelial growth are very important, both biologically and economically. Up to the present time, various authors have studied the effects of temperature on different kinds of fungi, but as far as the writer is aware, this relation on the species of *Valsa* has not yet been investigated.

I have studied it on our fungus, using the synthetic solution of the following formula for the culture medium.

Potassium biphosphate	0.25 gr.
Magnesium sulphate	0.25 gr.
Glucose	10 gr.
Asparagin	10 gr.
Redistilled water	1000 cc.

Took 50 cc. of the solution in each Erlenmeyer's flask of 200 cc. Sterilized, and inoculated with a drop of redistilled water, containing numerous pycnospores. Incubated them at the various temperatures. After two weeks, strained the mycelium through the filter paper of known weight, to compare the dry weight of the mycelium at every temperature. The results are shown in the following table:—

Table 22. The effect of different temperatures on the growth of the causal fungus. Weight is the average of 3 flasks.

Temperature	Dry weight of mycelia (gr.) (average of 3 flasks)
2° – 5°	0.0000
10° – 12°	0.1108
15° – 16°	0.1268
18° – 20°	0.1807
24° – 25°	0.1855
27° – 28°	0.2064
30° – 32°	0.2306
34° – 35°	0.1619
38° – 40°	0.0000

At the temperature 2° – 5° C., the fungus did not show any visible growth, but maintained its vitality and after two weeks, when incubated at 25° C., the mycelium grew vigorously. However, at 38° – 40° C., the fungus died, and though incubated at 25° C., no visible growth took place.

Experiment 2.

In this case, I have used the culture solution similar to that used in Experiment 1, in which 15 grams of cane sugar were used in place of 10 grams of glucose, and 10 grams of peptone instead of 10 grams of asparagin.

Table 23. The effect of different temperatures on the growth of the causal fungus, in solution containing cane sugar and peptone.

Temperature	Dry weight of mycelia (gr.) (average of 3 flasks)
15° – 16°	0.1202
18° – 20°	0.1088
25° – 26°	0.1944
28° – 29°	0.2567
29° – 30°	0.2094
33° – 34°	0.1296
37° – 38°	trace

From the above two experiments, it may be safely inferred that the optimum temperature for the growth of the fungus is 28° – 32° C., the maximum temperature about 37° – 38° C. and the minimum somewhere between 5° – 10° C.

XIV. Thermal Death Point of the Pycnospores.

The wet heat treatment was used in this experiment. Glass tubes having the diameter of 1 cm. were filled with about 5 cc. of sterilized redistilled water, heavily suspended with the pycnospores. The tubes were incubated at the required temperature in a self-regulating water bath under a slow mechanical agitation, with a certain intervals of time. The spore suspensions were removed to the hard agar media of potato (P), potato-cane sugar (1 %) (P_1), corn meal-cane sugar (1 %) (C_1), soy

(after Miyoshi) (S), apricot (A) and apple bark decoction (B); Then they were incubated at 25° C. The results were as follows:—

Table 24. Thermal death point of the pycnospores.

Intervals	Temperature	P	P_1	C_1	S	A	B
5 min.		±	+	±	−		±
10 min.	50°C.	−	+	−	−		±
15 min.		−	−	−	−		−
20 min.		−	−	−	−		−
20 min.			+	+	−	−	
30 min.		+	+	−	−	±	
1 hr.	45°C.	±			−		
1.5 hr.		−	+	−	−	−	
2 hr.		−	±	−	−	−	
3 hr.			−	−	−	−	
6 hr.		+			+		
8 hr.		±			+		
10 hr.		+	+	+	−		−
12 hr.	40°C.	±	−	±	−	+	−
14 hr.			+	±	−	−	−
16 hr.			+	±	−	−	−
18 hr.			+	±	−	−	−

As shown in the above table, the results were more or less different owing to the kind of the medium used. For instance, after 10 hours' exposure at 40° C., when the spore suspension were removed to the media of potato, potato-cane sugar and corn meal-cane sugar, the spores germinated and grew vigorously, but in those of soy and bark decoction, did not. However, if the spores were exposed for 15 minutes to the temperature 50° C., as well as for 3 hours to 45° C., they could not maintain their vitality. By heating at 40° C. for 18 hours, they retained their vitality on some media, while on the other media they could not.

XV. Morphological Difference between *Valsa Mali* and Related Species of *Valsa*.

From the results of my inoculation experiments, we may infer that our fungus seems to be specialized to the species of *Malus* and that under

favorable conditions, it has the power also to infect *Populus nigra* var. *italica*, *Salix sachalinensis* and *Prunus serrulata* var. *sachalinensis* and *P. yedoensis*, though in the next spring, these trees recovered from the disease. We must consequently take other species of *Valsa* which are naturally parasitic on these trees, into consideration, and compare them with our fungus.

a. Comparison with *Valsa sordida* Nits.

Valsa sordida belongs to the same subgenus *Euvalsa* sect. *Microsporæ* as *V. Mali*. It is widely distributed in Europe, having been reported from Germany, Austria, Switzerland, Italy, France and Sweden. According to W. H. Long (22), it is a serious parasite causing the canker disease on ten species of Populus and three species of *Salix* in North America. Also in Hokkaido, this fungus is very common, parasitic on various species of *Populus*, but up to the present time, its ascus stage has not been discovered, and it is known only by its pycnidial stage, *Cytospora chrysosperma* (Pers.) Fr.

As previously stated, the cultural characters of this fungus isolated from *Populus nigra* var. *italica* are very different from those of *Valsa Mali*. Moreover, asci, ascospores and especially pycnospores of *Valsa sordida*, according to the description of the European and American authors, differ considerably in size and shape from those of the fungus under consideration. I will show these differences in the following table :—

	Valsa sordida	*Valsa Mali*
Asci	48—60 × 8μ (after Schröter) 40—45 × 8μ(after Ellis & Everhart) 30—40 × 5—7μ (after Traverso)	25—42 × 5.5—15.0μ Length : m31.505 ± 0.218μ σ ± 3.089μ Width : m8.946 ± 0.111μ σ ± 1.581μ
Ascospores	12 × 1.5—2.0μ (after Schröter) 8—10 × 1.5μ(after Ellis & Everhart) (in the Iowa sps.) 9—11 × 1.5μ (in the Krieger's sps.) 9—12 × 1.5—2.0μ (after Traverso)	7.0—11.0 × 1.4—2.1μ Length : m8.76 ± 0.0497μ σ ± 0.703μ Width : m1.671 ± 0.0079μ σ ± 0.112μ

	Valsa sordida	*Valsa Mali*
Pycnospores	4 × 1μ (after Schröter) 4 × 1μ (after Ellis & Everhart) 4 × 1μ (after Traverso)	4.0—10.0 × 0.8—1.7μ Length : m5.856±0.036μ σ±0.816μ Width : m1.305±0.061μ σ±0.137μ

b. Comparison with *Valsa ambiens* (Pers.) Fr.

This fungus belongs to the subgenus *Euvalsa*, sect. *Macrosporæ* and is widely distributed throughout Europe and North America on various kinds of broad-leaved trees including apple trees. In Japan, it has been found on *Morus*, *Malus*, *Salix* and probably more. The facts that the lower half of the stroma consists mostly of the host tissues, and the shape of asci is oblong or clavate, and that the size of pycnospores is 5—7 × 1μ (after Schröter), seem to show a close relation to our fungus, but the measurements of the asci and ascospores are very different from those of our fungus.

	Valsa ambiens	*Valsa Mali*
Asci	8 spores, rarely 4 spores in an ascus 40—55 × 12—15μ (after Ellis & Everhart) 40—88 × 8—16μ (after Schröter) 40—88 × 8—16μ (after Traverso)	Always 8 spores in an ascus 24—42 × 5.5—15.0μ Length : m31.505±0.218μ ±3.089μ Width : m8.946±0.111μ σ±1.581μ
Ascospores	14—24 × 3—5μ in 8-spored asci 24—36 × 5—8μ in 4-spored asci (after Ellis & Everhart) 16—24 × 3—6μ in 8-spored asci 24—36 × 5—8μ in 4-spored asci (after Schröter) 14—24 × 3—5μ in 8-spored asci 22—32 × 5—7μ in 4-spored asci (after Traverso)	7.0—11.0 × 1.4—2.1μ Length : m8.76±0.0497μ σ±0.703μ Width : m1.671±0.0079μ σ±0.112μ

c. Comparison with *Valsa japonica* Miyabe et Hemmi.

This fungus was carefully studied by T. Hemmi (13) and he proved that it is parasitic in Hokkaido on *Prunus yedoensis*, *P. serrulata* var. *sachalinensis*, *P. Mume* and *P. Persica*.

As shown in the following table, it is clearly distinct from our fungus in many morphological characters.

	Valsa japonica	*Valsa Mali*
Size of stroma	3 – 5 mm. in breadth	1.2 – 2.0 mm. in diameter
	2 – 4 mm. in height	About 1.0 mm. in height
Number of perithecia in a stroma	Commonly 15 – 40	3 – 14
Asci	Cylindrical or rarely clavate	Clavate or oblong, round or truncate at the apex
	60–96 × 8.8–16μ	24–42 × 5.5–15.0μ
Ascospores	18–22 × 4.0–4.8μ	7.5–10.0 × 1.5–1.8μ
Color of spore-horn	Red	Yellow

d. Comparison with *Valsa leucostoma* (Pers.) Fr.

This fungus is the type of the subgenus *Leucostoma* and a distinct different species from *Valsa Mali*. Throughout Europe, Australia and America, it causes the disease known as the "die back" on apple, peach, plum, almond, apricot, cherry trees, etc. In the middle and southern Japan, this fungus seems to cause a serious damage to peach and plum. However, as yet, no one has ever found it on apple trees in our country. In 1921, L. H. Leonian (20) reported from New Mexico the breaking out of an apple canker caused by *Valsa leucostoma*. His descriptions of the disease and its causal fungus seems to coincide rather better with those of the Japanese Apple-canker caused by *Valsa Mali* than those of the canker caused by *Valsa leucostoma* as described by F. M. Rolf (35) and others.

XVI. General Summary.

1. A great number of apple trees throughout Hokkaido and the Northern Provinces of Japan are badly affected with a canker disease caused by *Valsa Mali* Miyabe et Yamada.

2. Any organism except the hyphae of *Valsa Mali* has not been

isolated from the water-soaked or cankered lesion in Hokkaido and Aomori Prefecture. *Bacillus amylovorus* has no relation to this apple canker.

3. Generally, the infected bark shows at first a swollen water-soaked appearance in early spring without exuding any fluid drops, later the lesion dries up and becomes sunken, and about a month after infection the black stromata of the fungus are formed over the entire cankered area.

4. The causal fungus hibernates in the bark with pycnidia or perithecia, or even with mycelia only without fruiting bodies.

5. Among the varieties of apple trees in these districts, there are none resistant to the disease under every ordinary condition. However, Stark Florence and Newtone, which have no economic importance, are not affected by the fungus.

6. Under favorable conditions, *Valsa Mali* has the power to infect *Populus nigra* var. *italica*, *Salix sachalinensis*, *Prunus serrulata* var. *sachalinensis* and *P. yedoensis*, when they were inoculated with a piece of the hyphae on the artificially made wounds. When inoculated with the pycnospores, infection did not take place in every instance. All affected trees, however, recovered from the disease in the course of a year. In the case of *Malus Zumi*, infection took place when it was inoculated either with the pycnospores or mycelium, and the canker progressed without showing any sign of recovery.

7. There are many oil drops in the pycnospores and ascospores, as well as in the asci and hyphae of the fungus.

8. The germination of the pycnospores and ascospores is preceded by an enormus swelling of the cells, and both spores are often divided into two cells before the germination occurs.

9. The most vigorous growth of the mycelium, and the abundant production of pycnidia have taken place on the culture media of apricot agar and sterilized apple twigs.

10. The more we increase the amount of cane sugar or glucose, the more we get the stronger mycelial growth. In the cases of peptone and asparagin, 1 percent media are most suitable for the growth of the fungus. The combination of glucose and asparagin is most advisable for the purpose of the pycnidial production.

11. The media containing lower concentrations than M/2000 of tannic acid show more luxuriant mycelial growth than without it. But on the media which contain higher concentrations than M/1000, the growth of the fungus is retarded.

12. The optimum temperature for the growth of the fungus is 28°–31° C., the maximum temperature about 37°–38° C. and the minimum somewhere between 5°–10° C.

13. The pycnospores of the fungus can not retain their vitality after 15 minutes' exposure at 50° C., as well as 3 hours' at 45° C.

XVII. Literature Cited.

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XVIII. Explanation of Plates.

Plate XXVII.

Valsa Mali, Miyabe et Yamada.

Fig. 1. Asci and ascospores. (× 1200).
Fig. 2. Conidiophores and pycnospores. (× 1200).
Fig. 3. Oil drops in asci and ascospores stained with Sudan III. (× 1200)
Fig. 4. Oil drops in pycnospores stained with osmic acid. (× 3000).
Fig. 5. Oil drops in germinating swelled up ascospores stained with alkannin. (× 1200).
Fig. 6. Oil drops in germinating swelled up pycnospores stained with Sudan III. (× 1200).
Fig. 7. Oil drops in mycelial cells in cultures stained with alkannin. (× 1200)
Fig. 8. Germinating process of pycnospores. (× 650).
a. Resting spore. b. 25 hours after sowing. c. After 37 hours. d. After 49 hours. e. After 52.5 hours. f. After 56.5 hours. g. After 61 hours. h. After 64 hours. i. After 72 hours. j. After 75.5 hours. k. After 80.5 hours.
Fig. 9. Two celled ascospores just before germination. (× 650)
Fig. 10. Germinating ascospores. (× 650)

Plate XXVIII.

Valsa Mali, Miyabe et Yamada.

Fig. 1. Cross section of a pycnidium.
Fig. 2. Cross section of perithecia.
Fig. 3. Mycelia in the sap-wood of the apple tree.
Fig. 4. Oil drops in the hyphal cells in the vessels of the host tissue.

Plate XXIX.

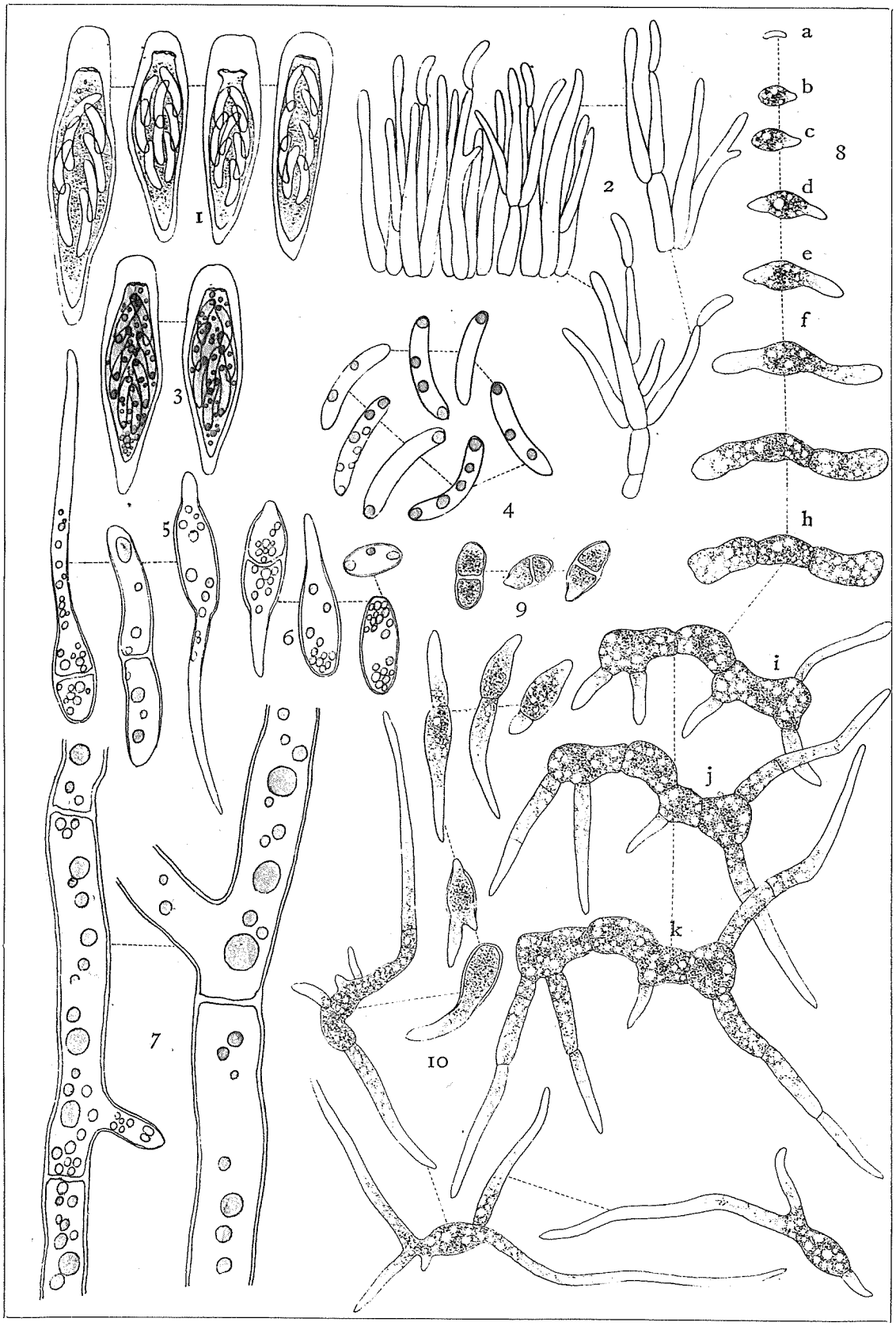
Fig. 1. Outlined canker, indicating half a month growth. Yellow Transparent.
Fig. 2. *Ditto.* Wealthy.

Fig. 3. Diseased apple branch, showing some stromata oozing out spore-horns.
Fig. 4. Outlined canker, indicating half a month growth. Fameuse.
Fig. 5. *Ditto.* Northern Spy.
Fig. 6. *Ditto.* White Pippin.

Plate XXX.

Fig. 1. Canker disease of *Salix sachalinensis* caused by *Valsa Mali.*
Fig. 2. Canker disease of *Populus nigra* var. *italica* caused by *Valsa Mali.*
Fig. 3. Canker disease of *Prunus yedoensis* caused by *Valsa Mali.*
Fig. 4. Canker disease of *Prunus serrulata* var. *sachalinensis* caused by *Valsa Mali.*
Fig. 5. Canker disease of *Malus Zumi* caused by *Valsa Mali.*
Fig. 6. Recovered *Populus nigra* var. *italica* from the canker disease inoculated with a bit of the mycelium of *Valsa Mali* on Sept. 13, 1922. (Photo. July 27, 1923).
Fig. 7. Recovered *Prunus yedoensis* from the canker disease inoculated with bit of the mycelium of *Valsa Mali* on Sept. 13, 1922. (Photo. July 28, 1923).

Contents.



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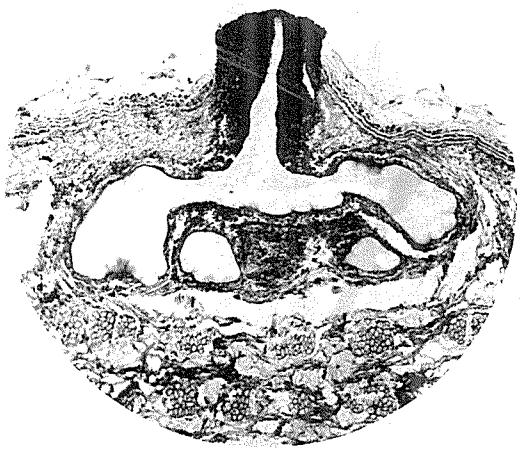

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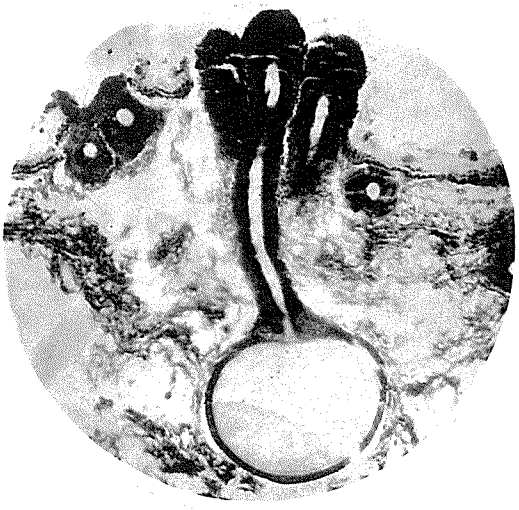

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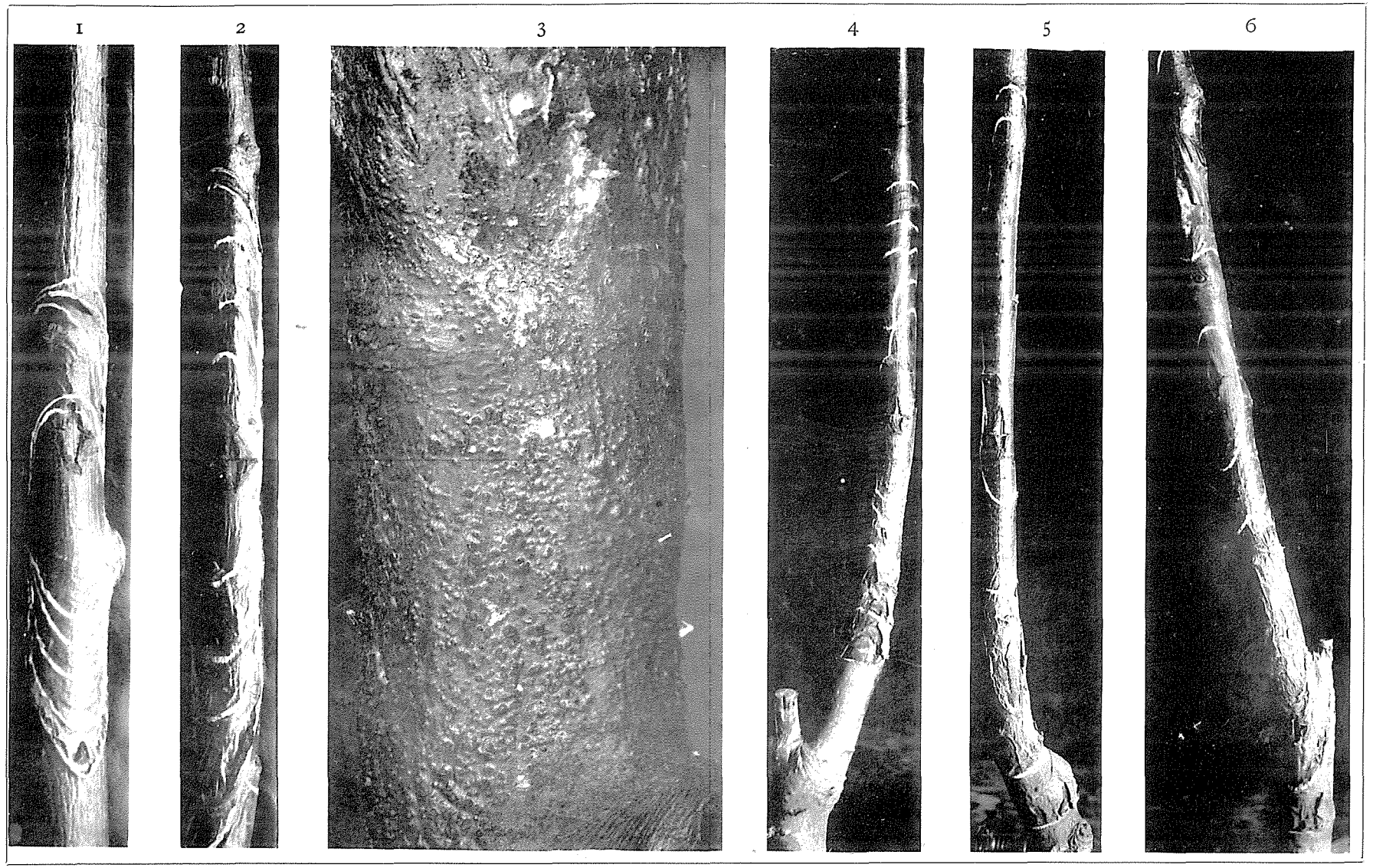

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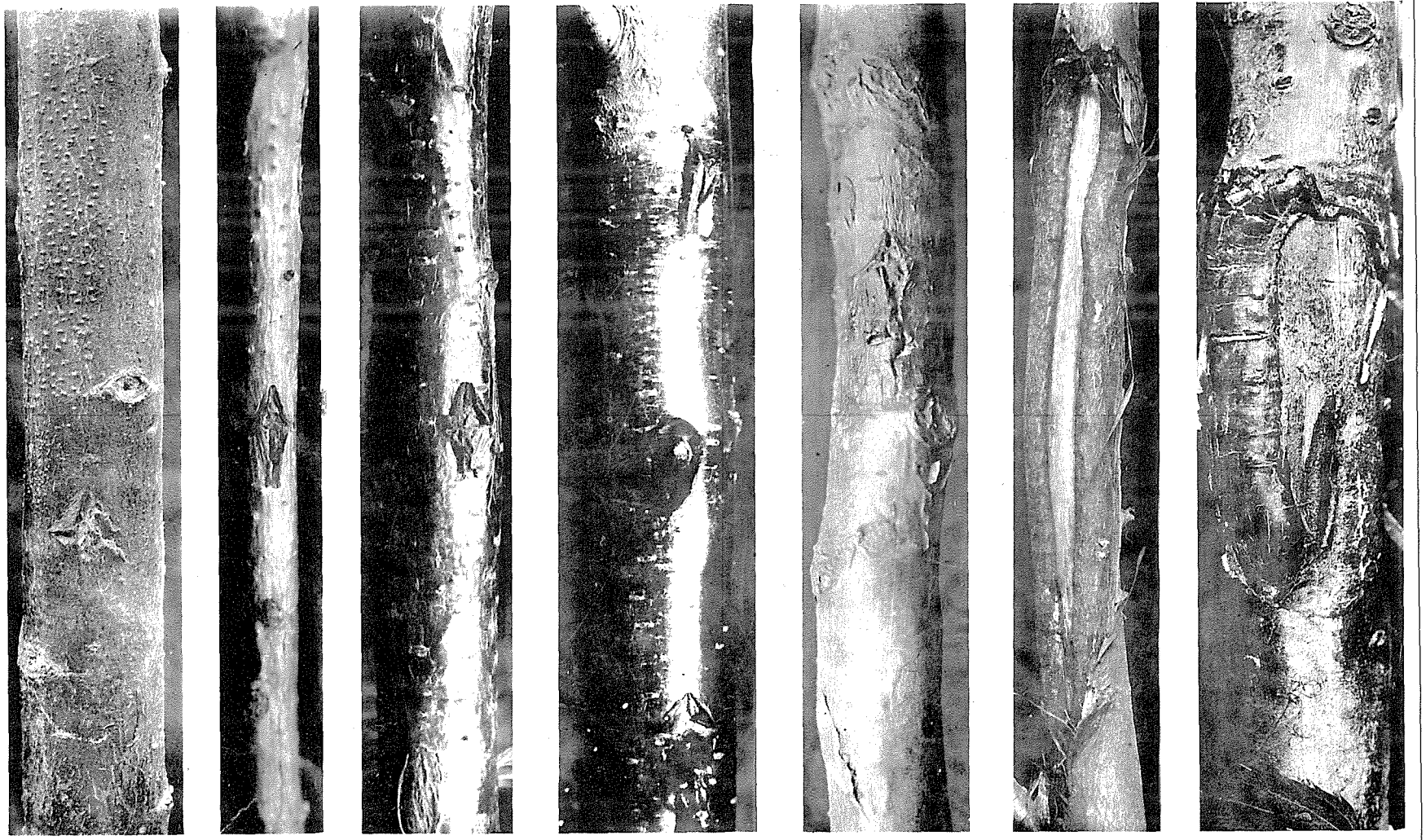

K. Togashi, photo.

Errata.

(Jour. Coll. Agr., Hokkaido Imp. Univ. Vol. XII. Pt. 3)

Page	line	For	read
268	11 from below	indicatiom	indication
269	10	pyconospores	pycnospores
„	14	Pl. II.	Pl. XXVIII.
„	1, 17 from below	Pl. I.	Pl. XXVII.
270	8	Pl. II.	Pl. XXVIII.
„	15	60–30	60–80
„	12 from below	Pl. I.	Pl. XXVII.
„	11 „ „	28–35×7.0–10.5	24–42×5.5–15.0
„	2 „ „	7.5–10.0×1.5–1.8	7.0–10.0×1.4–2.1
271	5, 18	Pl. II.	Pl. XXVIII.
„	18	Pl. I.	Pl. XXVII.
„	20	Pyconospores	Pycnospores
„	2 from below	3.0–10.0	4.0–10.0
272	4 „ „	± 1.169	± 1.166
„	6 „ „	5.890	5.860
274	1 „ „	21	12
303	2 „ „ in 3rd. row	++++	+++
314	11 from below	25–42×	24–42×
315	13 „ „	± 3.089μ	δ ± 3.089μ