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Short Title

Biology of *Plasmopara halstedii* on sunflower

BIOLOGY AND EPIDEMIOLOGY OF *PLASMOPARA HALSTEDII* ON SUNFLOWER

by

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I - INTRODUCTION

The fungus responsible for the downy mildew disease, common on commercially grown sunflowers, is Plasmopara halstedii (Farlow) Berl. et de Toni, one of the Peronosporaceae in the Oomycetes, known to be soil borne and to infect via the roots.

World wide in distribution, the fungus is coextensive with its main hosts, the commercial varieties of the sunflower, Helianthus annuus L. This plant is mainly grown for its seeds, which should more correctly be referred to as achenes, being the seed proper with the pericarp around it. The achenes are used for birdseed, and in eastern Europe for human consumption, but particularly for the production of a vegetable oil. Sunflowers are sometimes grown as a forage crop as well. It is a common crop in eastern Europe and South America, though frequently found in other parts of the world. Leppik (1962) gives a distribution map of the fungus, showing that it occurs in Europe, the Middle East, Asia, the Far East as well as South America and parts of Africa. In the United States it is widespread. In Canada it is common in Manitoba, whilst in Quebec it has been reported as a limiting factor near La Pocatière, where sunflowers can no longer be grown for forage.

That the disease can be severe was shown by Yagodkina (1956), who reported that 76 % of the 133.500 hectares planted with

sunflowers in 1955 in the Krasnodar region of southern Russia was infected with Plasmopara. In individual fields, an average of 3.2 % of the plants was infected, with maxima of up to 60 % in some fields. In Manitoba also, infections of up to 60 % have been reported (Sackston, 1954).

In most cases an infected plant will not form any useful seeds, and consequently is a total loss. The neighbouring plants may benefit from the extra space available to them, but this will not make up for the lost potential. The disease is often found in many consecutive seedlings in a row, as infestation of the soil mostly occurs in patches, presumably originating from remains of one infected seedling in a previous year. As the percentage of infected plants corresponds roughly with the losses suffered, it can be seen from the infection percentages given above, that the disease can be of great economic importance. The survival of the oospores for a number of years is another factor contributing to the economic importance of the disease, since survival for at least three years has been reported (Anon., 1952), which makes it necessary to grow other and perhaps less desirable crops over an extended period of time.

The pathogen responsible for the downy mildew of sunflowers is of interest, because of the unusual combination of features that make up its lifecycle, and the etiology of the disease it causes. There are other fungi in the Oomycetes that

are obligate parasites which can survive in the soil for many years, as for example Plasmodiophora brassicae Woronin which causes clubroot of crucifers or Synchytrium endobioticum (Schilb.) Perc. responsible for the wart disease of potato. But they will usually infect only the underground portions of the host. On the other hand, Plasmopara halstedii will not only spread into all parts of the plant, but will produce spores on the leaves as well. It is believed that the sunflower roots are usually infected by the zoospores formed when the oospores germinate.

Since Plasmopara can form spores that will infect the roots of other seedlings, theoretically, it could spread much faster over longer distances, than the majority of soil born diseases. It is not known if this really happens to any extent under field conditions. By the time spores have formed in an infected field, all the sunflower seedlings in the same area will probably have developed to the point where, if they become infected, the fungus cannot spread far enough to cause severe infection and visible symptoms. However, the soil in which they are growing could become infested by the roots that are left behind in the field at harvest time. Should this be the case, it might provide another explanation for the fact that the disease is so common in some areas, and not in others. Most workers have been unable to find evidence for seed transmission of the disease (Nishimura, 1922, Spekar, 1952), and although Novatelnova (1963) proved that the

disease could be transmitted by seed on a local scale, this will probably prove not to be the major method of spread of infection.

Field tests to determine the resistance of varieties and selections produced in Manitoba, had been conducted at the Experimental Farm in La Pocatière, but were slow to give results. The first object of this present project, therefore, was to find a faster and simpler method of determining the resistance of different varieties of commercial sunflowers to Plasmopara.

When the whole plant is penetrated by the fungus, a pronounced stunting often occurs, and the leaves may show malformations that resemble those caused by certain hormonal herbicides. The second object of this present project, therefore, was to try to determine if hormone-like substances play a role in causing this symptom, for although the symptoms were severe in the leaves of most stunted plants, some are strongly stunted before the first of these symptoms become visible, in the form of yellow areas along the veins. In the latter case it seems hard to explain stunting by the theory that the plant would be unable to provide food material both for itself and for the pathogen.

Other objects of this study included: the influence of temperature and of light on the development of the disease, the survival of zoosporangia under different conditions, and how long after planting the first oospores are formed in the roots of seedlings.

II - LITERATURE REVIEW

According to Leppik (1962), P.halstedii, like its main host Helianthus, can be considered as being American in origin. The first record of the occurence of the fungus was made by Farlow in 1883, on plants from the United States, who stated that conidia of the fungus germinate to produce zoospores, but that he thought it unlikely that they could penetrate the uninjured upper surfaces of mature sunflower leaves.

The first detailed study of the fungus, and the disease it causes, was made by Nishimura (1922) in Japan. He found that the fungus generally originated in the underground portions of the plant, and subsequently spread into the aerial parts. He showed that seedlings would become infected when planted in soil originating from plots where diseased plants had grown before and also in soil that was infested with zoospores immediately before sowing the seeds. Nishimura also found that entry of zoospores could take place through the uninjured epidermis of the roots, although the fungus penetrated more readily by way of broken root hairs and small injured areas. He described the presence of mycelium in the intercellular spaces, and the development of oogonia and antheridia which preceded the formation of oospores. In Helianthus divaricatus, he stated, the infection could take place through stomata of the leaves, and the fungus could travel down to the rhizome. Later he

also described the haustoria of the fungus (Nishimura 1926).

In 1927 Young and Morris, working in the United States, thought it almost certain that Plasmopara survived the winter in diseased sunflower seeds. Their attempts to inoculate the uninjured upper surfaces of leaves with conidia all failed. Also in the United States, Young, Jellison, and Morris (1929) detected the hyphae of the fungus in sections of the roots, stems, cotyledons and the leaves of infected plants, often within a week after emergence. They reported that severely infected plants lived only a few weeks, and that survivors produced no viable seeds.

In Russia, Yagodkina (1956) reported on the occurrence of the fungus in the Krasnodar region. She stated that P.halstedii was not found on weeds of the Compositae in infected fields near diseased sunflowers, not even on other species of Helianthus, and interpreted this to mean that there is a new race of Plasmopara in the area, possibly even a new species. Novatelnova (1960) claimed to have distinguished three different forms of the disease: forms 1 and 2 being characterized by a diffuse distribution of the fungus in the plant, and form 3 giving a localized infection. The fungus was said to develop best in young growing tissues, and she suggested that this may be due to the higher concentration of growth stimulating substances there, as well as a more active respiration; she thought that stunting may be the result of this preference for meristematic tissues, with consequent suppression of growth.

In 1962, Novatelnova (1962 a) claimed that P.halstedii was not a single species, but comprised a number of separate species, each with its own distribution area and distinctive sporangiophore features, as well as spore characteristics. She postulated that within the P.halstedii complex there would be a P.helianthii infecting only Helianthus species. It would include three biological forms:

f. helianthi with an annual mycelium occurring only on the annual species of Helianthus.

f. perennis with a perennial mycelium on perennials of the same genus, and

f. patrens which would also attack perennials, but differing in its sporophore morphology.

P.halstedii occurs in many different parts of the world. A report on the distribution of this fungus on a world-wide scale was compiled by Leppik (1962), and published by the FAO. In South America Sackston (1957) found the disease on sunflowers in Uruguay and Argentina. Podhradszky (1954) described Plasmopara as a new disease of sunflowers from Hungary. In 1952 Spekar reported from Yougoslavia that after World War II the downy mildew disease of sunflowers was found in Slovenia and Serbia. Nicolic (1952), also from Yougoslavia, gave details of symptoms and the development of primary and secondary infections.

The first report of P.halstedii from Canada, was that of Bisby and Conners (1928), who described it as a plant disease new to Manitoba. In 1949 a stunting of sunflowers, and a possible connection with the downy mildew fungus, was reported by Sackston. He also reported that the disease was severe in Manitoba in 1953 and 1954 (Sackston 1954, 1955), and suggested that this might have been associated with the high moisture of these two years. In the dry 1955 and 1956 summers, only trace amounts of Plasmopara infection could be found in Manitoba (Sackston 1957). Work with the downy mildew of sunflowers was reported from the Canada Department of Agriculture Experimental Farm at La Pocatière (Anon. 1949), and by Perrault in 1952. The latter found that oospores survived in the soil for three years. In 1956 it was reported from La Pocatière (Anon. 1956), that the fungus had been found in the roots of two compositae, the dandelion and ragweed.

Sackston (1955), also reported that the infection was systemic and caused moderate to severe stunting. Certain symptoms were compared with those caused by 2-4 D treatment, and were described as distortions and rugosity of the leaves. The question of the nature of the physiological relationship between the infection of the plant and the stunting which follows, was also raised by Novatelnova (1960), when she pointed out that the fungus has a preference for young tissues, and suggested that this might be due to a higher concentration of growth substances there.

Grieve (1943) found stunting of virus infected plants to be associated with a decrease in the assimilatory efficiency of leaves, and destruction of growth hormones. Srivastava, Shaw and Vanterpool (1962), working on the effect of Albugo candida on growth substances in Brassica napus, found smaller amounts of indol-acetic-acid, (IAA), and other growth substances, in diseased inflorences than in healthy ones. Daly and Inman (1958), working on changes in auxin levels in safflower hypocotyls infected with Puccinia carthami, obtained more IAA from diseased than from healthy plants. They studied the respiratory metabolism during pathogenesis, and suggested that metabolic events can be separated into two phases. In the initial stages of infection, the parasite induced a change in the key growth-controlling processes of the invaded cells, causing an increased rate of host metabolism. In this phase the host furnished metabolites for growth of the pathogen, leading to a rapid development of the fungus. In the second phase, as the mycelium increased in amount, its contribution to the total metabolic activity of the infected tissues gradually supplanted that of the host. Daly and Inman warned, however, against a tendency to implicate changes in IAA levels as a primary cause of variation in normal and abnormal growth, because of recent recognition of the possible importance in higher plants of growth inhibitors, modifiers, and previously unknown hormones.

III - MATERIALS AND METHODS

The sunflower seeds used for these experiments were the varieties CM5, Sunrise, and S37-388RR, which had originally been obtained from the Canada Department of Agriculture Research Station, Morden, Manitoba, and which were also being used simultaneously in other projects of the Department of Plant Pathology, Macdonald College. In three experiments, a series of nine Russian varieties were used, that had been supplied in order to test their resistance to the Canadian strain of P.halstedii as used for the present work, as well as to other fungi being studied at Macdonald College.

Early in this work, it was found that the seed of the variety Sunrise had a very low germination rate, since it was several years old. In addition, many of the seeds that germinated gave rise to deformed plants. This material was therefore multiplied in a field plot, and the fresh seed used in subsequent experiments.

The varieties CM5 and Sunrise, as well as the Russian ones, proved to be highly susceptible to Plasmopara, with up to 100 % of the seedlings tested being infected. The variety S37-388RR (RR standing for the Rust Resistance for which it was selected) proved to be highly resistant. However, under ideal conditions a few plants could be infected. When 100 % of a series of CM5 or Sunrise plants was infected, as much as 5 % might be infected in

S37-388RR, as measured by the number of plants on which sporulation of the fungus could be induced. Also, the amount of sporulation on the leaves and cotyledons of infected plants was much less in S37-388RR than in CM5 and Sunrise.

A few experiments were carried out using the wild sunflower species Helianthus maximiliani Schrad., H. laetiflorus Pers., H. tuberosus L, and H. subtuberosus Bourgeau. These plants had been obtained from Winnipeg in 1961, and put into permanent plots in the fields of Macdonald College. Seeds were harvested in the fall of each year after the first frost. Seedlings grown from that seed, as well as plants grown from sideshoots collected in these plots, were used in greenhouse experiments.

Plasmopara halstedii has been present for many years on sunflowers at La Pocatière, Que., and the Experimental Farm there has plots where year after year varieties have been tested for their susceptibility, and where the soil is thus heavily infested with the fungus. Several bushel bags of this soil had been sent to Macdonald College in 1961, 1962, and 1963, and the first infected plants were obtained from seeds sown in this soil. Since the soil was a heavy clay, and since some lots were collected while the soil was wet, it was very hard and lumpy on arrival and difficult to handle. In some of the experiments, sand was mixed with the clay in an effort to prevent the soil from becoming too solid in the pots. The seeds were sown in 4" pots and kept in the

greenhouse or in controlled environment cabinets. Seedlings developing symptoms did so from 2-6 weeks after seeding.

Sporulation could be induced in a minimum of 10 days after seeding and infection, though 14 days was more commonly used as the amount of spores obtained would be much higher. The plants were found to be at the right stage for induction, when the cotyledons started to look somewhat grayish and shrivelled, and the seedlings started to fall over, indicating that their roots had been destroyed. It was then that the maximum amount of spores could be obtained. This falling over could be termed the damping-off stage of the downy mildew disease. At the first sign of the damping-off, the plants are placed in a cabinet, roughly 125 cm long and wide and 100 cm high, where the temperature could be controlled at 20°C. A humidifier was kept running overnight. Spores were formed after the humidifier had been running for only six hours, although water would stay on the leaves for a much longer period of time, if the light was turned off at least half an hour before the humidifier. Best results were obtained by running the humidifier all night, that is to say for at least 12 hours. Sometimes the cabinet was not available, and an improvised cover of plastic sheets in the greenhouse proved to be almost as effective so far as the humidity was concerned. However, the temperature in such a small space, completely closed off from the outside air, could rise to very high levels on

sunny days, so that this technique could only be used at night. After the formation of spores had been induced overnight, whether in the cabinet or in the improvised facilities, the plants were exposed to the greenhouse atmosphere once more, to allow them to dry, before the cotyledons and the leaves bearing the spores were collected.

When the fungus was induced to sporulate on the cotyledons and leaves it had penetrated, the area covered by the spores was found to coincide almost completely with the area of penetration of the fungus. Occasionally spores were formed on the stem and the petioles, and sometimes zoosporangia were found in the pith when the stems were sectioned.

At first dry spores were collected by brushing them off the leaves into a water-filled petri-plate, making a spore suspension which could be diluted to any desired concentration. However, the spores floated very readily in the air, and many were thus lost. In addition these floating spores constituted a danger to other sunflowers in the area where the spores had to be collected. Therefore, a new method had to be devised. Leaves and cotyledons covered with spores were clipped from the plants and collected in petri-plates. A number of them were then placed in a glass beaker and shaken with tap water in order to obtain a spore suspension. This proved to be highly satisfactory, since the leaves did not have to be dry, as in the brushing method, and the possibility of contaminating other plants was greatly reduced.

Attempts were made to keep the petri-plates containing the sporulating fungus for later use in the preparation of spore suspensions. At room temperature there was spoilage and loss of infectivity within one week; storage in a refrigerator extended this period to two weeks; while storage in a freezer proved much more successful, as there was little loss of infectivity over a period of up to 3 or 4 months. This meant that spore suspensions could be prepared at any time, avoiding the necessity of having to have infected seedlings on hand at the right stage, whenever fresh spores were required. P. halstedii is an obligate parasite and cannot as yet be grown on artificial media.

Ready made spore suspensions were sometimes kept in medicine bottles, stored in the freezer, and proved to be infective up to 6 weeks after preparation. However, as these bottles occupied more space than leaves in petri-plates, and since they required several hours to thaw out and did not keep the spores viable for a longer period, it was not used as a standard technique.

The concentration of spore suspensions was determined with a haemocytomater, and was then adjusted to the concentration desired for the required experiment. In most instances where a known density of spores was desired, a concentration of 150.000 spores per cc was used.

A number of more specialized techniques used in only one or two experiments will be described later.

IV - SYMPTOMS AND ANATOMICAL FEATURES

The infection of sunflowers by P. halstedii can result in symptoms, that show up differently in the field than under greenhouse conditions. In the greenhouse even the healthy plants usually do not grow very high, the maximum reached being about 50 cm. They are also thin and spindly, compared with those in the field, and the leaves are very much smaller.

Seedlings growing in infested soil in the greenhouse might show symptoms, in the form of yellow areas along the veins, when they were three weeks old. The degree of infection varied however, in some plants narrow yellow areas would appear just along the main vein of one or a few of the leaves. In these plants growth might be slightly reduced when compared to the checks. In the seedlings that were more heavily infected, whole leaves would show the yellow areas that can be referred to as chlorosis (Figure 4). This indicated that Plasmopara had penetrated them completely, as sectioning of infested leaves had shown the margin of the yellow patches to coincide with the area penetrated by the fungus. Subsequently developed leaves would be invaded before they were full-grown, and eventually new leaves became smaller and smaller, as early invasion stopped their growth. In that case, the leaves were formed with progressively shorter internodes between them, resulting in stunting. It is not clear if this stunting is caused by interference with the growth

hormone mechanism, since starvation of the plant, due to competition for food by the large amount of mycelium present, can not be excluded as a possible factor. The infected areas constitute a large part of the photosynthetic surface of these seedlings. Nevertheless, such seedlings can survive for several weeks.

In plants growing in the field, symptoms are much more variable (Figures 1,2,3,4,5,6,7). In many plants they are analogous to those described for the seedlings in the greenhouse, although the plants are heavier and taller than those grown indoors. In some plants the symptoms did not show until the plants were 40 cm tall. The leaves of plants in the field are often more rugose than those of healthy plants. Most of the infested plants will still form flowers, even if they generally measure only 2-5 cm across, while the heads do not bend over the way those of healthy plants do (Figure 7). The internodes in these plants are much shorter than normal, particularly at the top, so that some stunting occurs.

Some plants in the field show interesting variations from this more or less standard reaction. In extreme cases the plant shows severe reduction in the length of the internodes, being only 50 cm high, in contrast to the usual 100 cm or more of apparently healthy plants, but having leaves of almost normal size, and showing no other symptoms, though usually developing them later (Figure 6). However, there are many inter-

mediate stages between this extreme and the more standard reaction, although the number of plants showing them is relatively small (Figure 5). After studying these cases, the starvation theory appears to become much less plausible. In these plants a disturbance of the normal growth hormone balance appears to be the most likely explanation.

Hand sections were made of various parts of diseased plants to study changes from the healthy ones, and how and where the fungus appears in the plants. In certain experiments all the roots of the plants were sectioned in order to determine if infection had actually taken place. Frequently the fungus was found to be present in the root, but to have failed to spread far enough upwards to lead to development of symptoms in the leaves.

In infected tissue, P. halstedii shows up as thin strands of mycelium in the intercellular spaces, and as haustoria in the surrounding cells (Figure 8). These haustoria are approximately round and vary in size from 10-25 μ, though young haustoria can be more elongated and then measure 5x10 μ. As they are usually present in all the cells around a certain intercellular space, the effect is very striking when seen under the microscope. Small round clusters of two to five haustoria can be found very readily, and because of this characteristic pattern, it is usually simple to distinguish a Plasmopara infection from one that might have been caused by another fungus. On a few occasions no haustoria could be found although mycelium was

present, but further up the plant, there were haustoria. Thus the mycelial strands must have been those of Plasmopara. In the few cases where only mycelium was present, it consisted of thin strands staining darkly in a cotton-blue lactophenol solution. In sections of the wild sunflower, Helianthus tuberosus, this was often the only indication for the presence of P. halstedii in the stem, although on the same plant the fungus was fruiting abundantly on the lower surface of the leaves. These strands must, therefore, have been the mycelium of Plasmopara.

By the use of hand sections, the extent of infection in individual plants was also studied. In wilting, two week old seedlings of a spore inoculated series, that were so severely infected that only the cotyledons had developed, the fungus was found to have penetrated into every part of the seedling. These plants were unable to grow any further and usually shrivelled and died rapidly.

The maximum diameter of the heads on diseased plants in the Macdonald College field plot was 10 cm, but most were between 2 and 5 cm (Figure 7). On the healthy plants, they varied in size from 20 to 30 cm. Very few seeds set in the heads of diseased plants, but those that did were all carefully collected. They were only 4-5 mm long and 2-3 mm wide, compared to the 8-10 and 4-6 mm respectively for seeds from healthy plants in the plot.

The hulls of the seeds from diseased plants were dark and discoloured. Nevertheless it is not at all certain that this discolouration was caused by P. halstedii, as many heads in the plot were attacked by other fungi. In any case, none of the 150 or so seeds, that were collected and planted showed any signs of P. halstedii infection on the seedlings, even after two months. These seedlings were much smaller at first than those that come from normal sized seeds, but after a few weeks they were nevertheless indistinguishable. That the discolouration of the hull might have been caused by Plasmopara, is indicated by the fact that in sections of the heads of diseased plants, this fungus could be seen to penetrate into the integuments and the embryo.

It is difficult to estimate the amount of fungus present in plants, since it must vary with the stage of development of the seedling, the part of the plant examined, and the variations that probably exist within individual plant organs. The only attempt along this line was with the degree of infection of roots. As the haustoria appear as separate clusters, it is easy to count them. Infections can be listed as light, moderate or severe respectively, when less than 10, from 10 to 50, or more than 50 clusters were present in any one section of the root examined. However, no results obtained with this method are described in the present work, since only a tentative approach to the problem of how to indicate the degree of infection was made.

Oospores could be found in sections of seedling roots as young as two weeks old, where inoculation had taken place when the seeds were planted. However, they represent a minority of cases. The oospores were round and had a hyaline wall when young, though later they became more yellowish in colour, and the wall became thicker and its surface rough. They measured 25 μ across (Figure 9).

Sometimes the formation of zoosporangia was observed in the pith of root and stem sections, where the pith was hollow. Normally the zoosporangia occur on the lower surface of the cotyledons and the leaves, although under certain conditions, they may also be formed on the upper surface of the cotyledons and on the lower parts of the stem.

Figure 1. Infected seedlings in which stunting has occurred early, and healthy plants.

Figure 2. Stunting of infected plants, extensive chlorosis in the upper leaves, rolling of leaf margins.

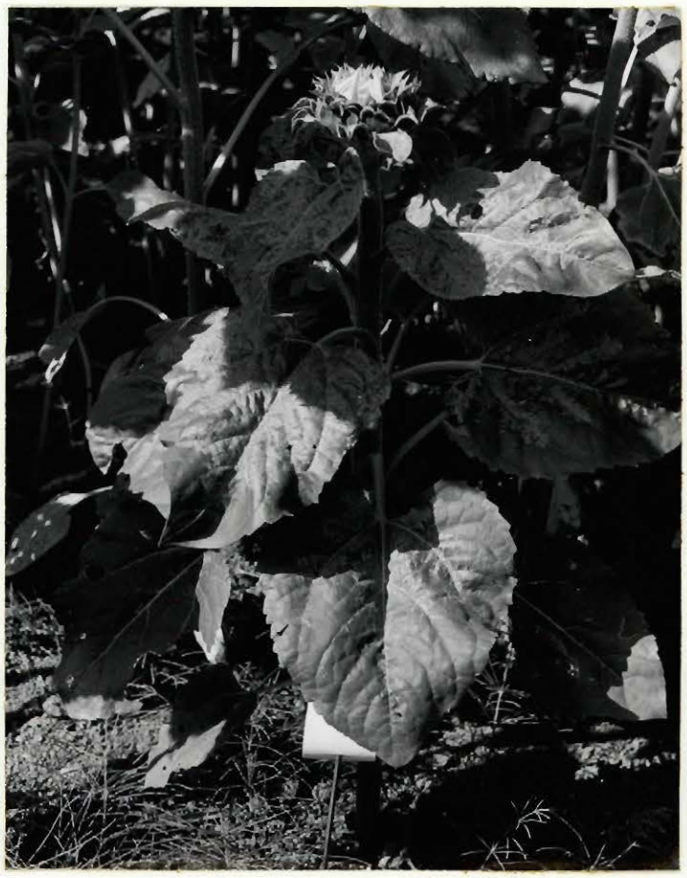

Figure 3. Chlorosis spreading along the veins, and into the lamina.

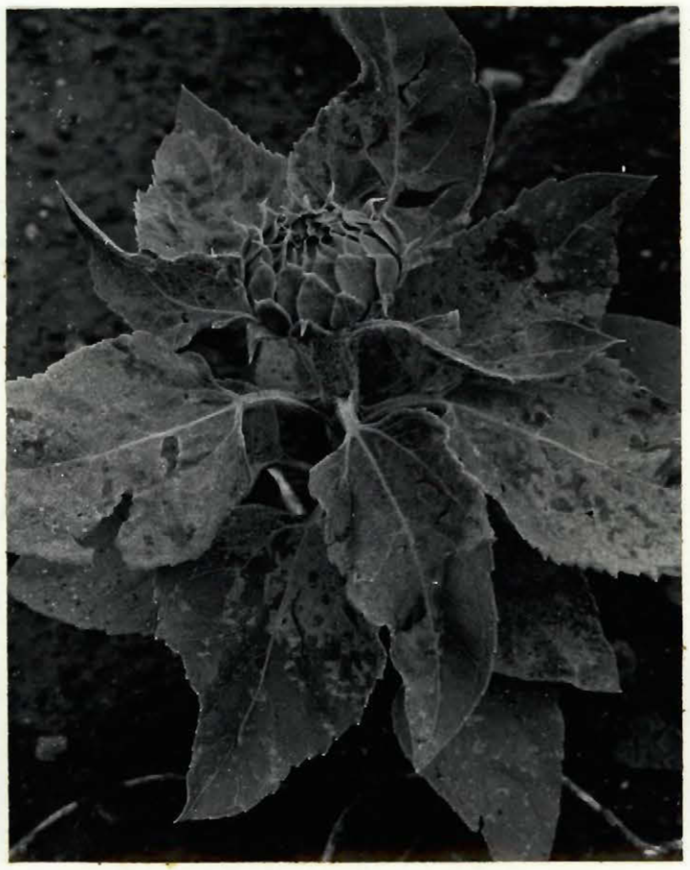

Figure 4. Chlorosis affecting most of the leaf.

Figure 5. Severe stunting with only light chlorosis along the veins.

Figure 6. Severe stunting, rugosity and leaf distortion, without chlorosis.

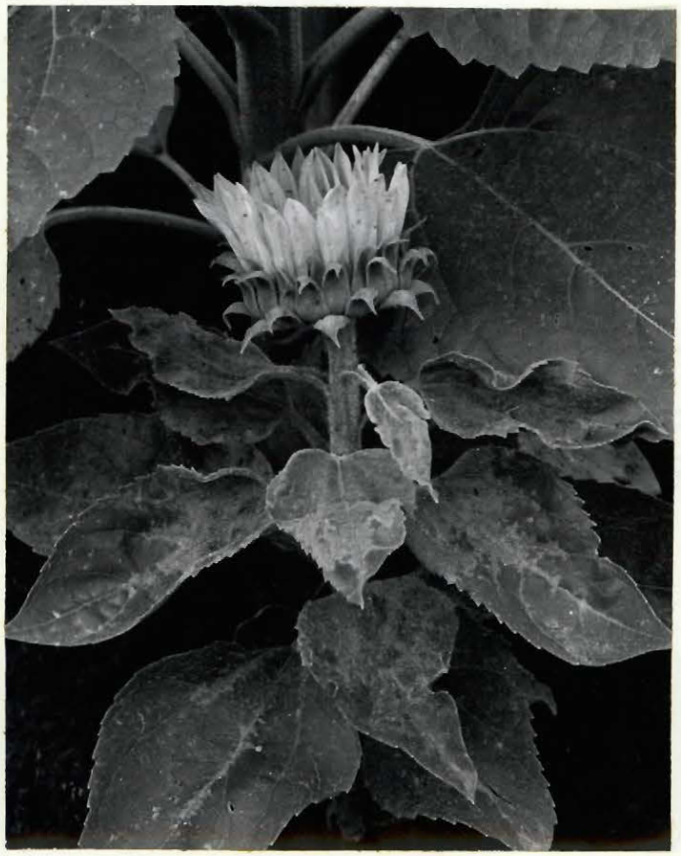

Figure 7. Flower on infected plant, not bending over.

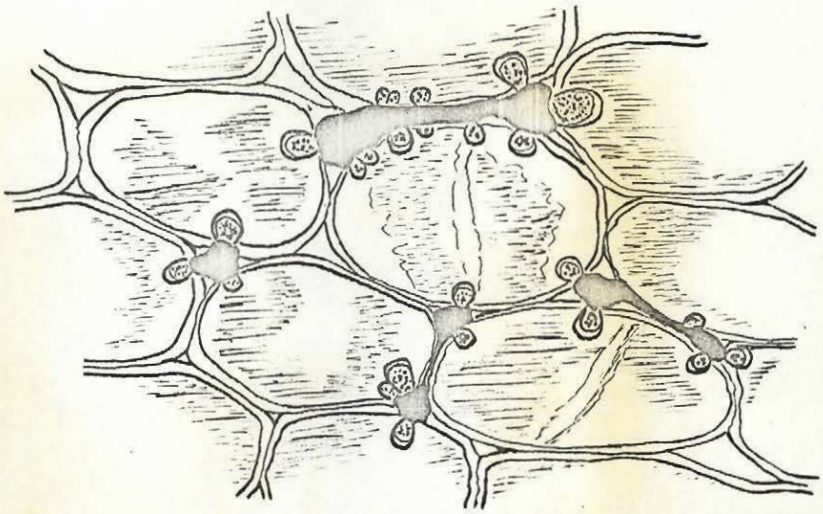

Figure 8. Intercellular mycelium, and haustoria of Plasmopara halstedii in a sunflower root section

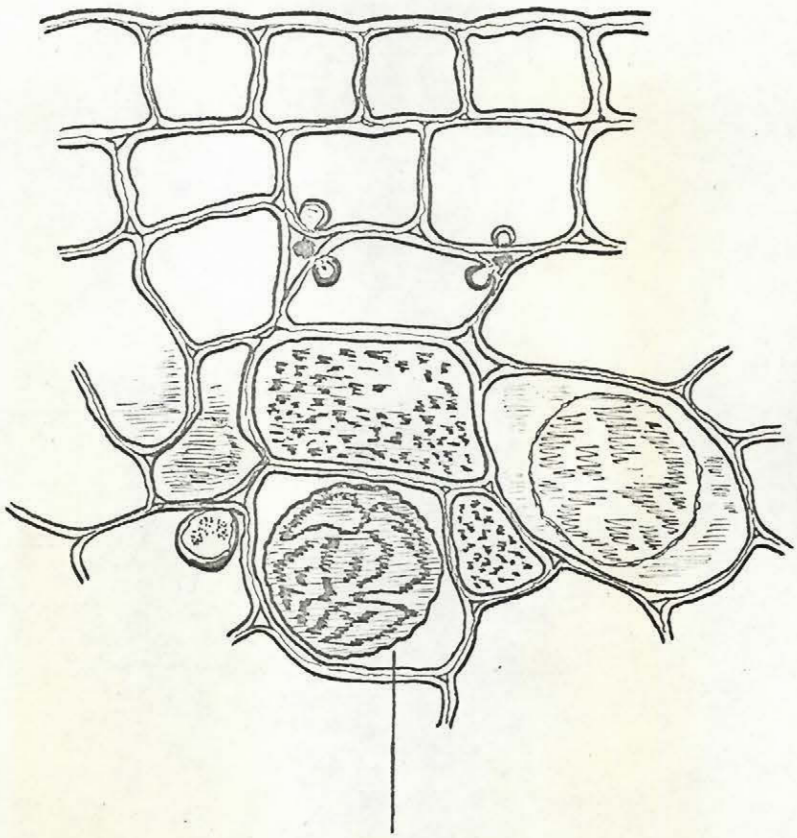

Figure 9. Oospores, and haustoria of Plasmopara halstedii in a sunflower root section

V - EXPERIMENTS WITH INFESTED FIELD SOIL

Experiment (i)

Sunflower seeds were sown in infested soil from La Pocatière in an attempt to get infected plants under controlled environmental conditions in the greenhouse and in controlled environment cabinets. Some seedlings contracted the disease, but the percentage that did so was never very high. The leaves of infected plants showed clearly delimited yellow areas spreading along the veins, and out to the margins. However, when the roots of all plants were collected, the presence of Plasmopara halstedii could be easily established, since the fungus forms distinctive haustoria in the cells surrounding the intercellular spaces in which the mycelium is present. Many of the plants thus found to be infected, did not show external symptoms. The age of the soil indicated in the tables is the period of storage under dry conditions at room temperature at Macdonald College. The results are presented in Tables I, IIa and IIb.

Experiment (ii)

According to Nishimura (1922), the fungus causing the infection of sunflowers growing in infested soil, originates from oospores which form zoospores when they germinate. Perhaps, therefore, oospores germinate after the sunflowers have started to develop, and may have been stimulated by substances given off

TABLE I

Plants showing symptoms in the leaves

Age of soil	Age of plants	Sunflower varieties								
		CM5			Sunrise			S37-388RR		
months	weeks	Inf.	Total	%	Inf.	Total	%	Inf.	Total	%
2	6	3	26	12	4	41	7	0	25	0
3	4	9	86	10	6	89	7	0	56	0
10	3	4	107	4	1	14	7	0	18	0
27	8	0	56	0	7	81	9	0	47	0

TABLE IIa

Plants with fungus in the roots, but no symptoms on the leaves, 6 weeks after sowing

Age of soil	Age of plants	Variety CM5		
months	weeks	Infected	Total	Percentage
3	6	46	81	56.8
27	6	19	42	46.0

TABLE IIb

Plants with fungus in the roots, but no symptoms on the leaves, 6 and 10 weeks after sowing

Age of soil	Age of plants	Sunflower varieties		
		CM5	Sunrise	S37-388RR
months	weeks	%	%	%
3	6	16	66	0
3	10	63	42	27
27	6	33	27	27
27	10	71	50	0

by the roots. An attempt was made to find out if sunflower seedlings influence the germination of the oospores. A second set of seeds was therefore sown in infested soil just after the first crop of seedlings had been grown in it for 6 weeks. The results are presented in Table III.

It was found that sowing sunflower seed in infested soil after other seedlings have grown in it, did not result in infections severe enough to produce external symptoms. The fungus became established in some of the roots, however.

From the data presented in Tables I, IIb and III, it can be seen that infection was more frequent in the CM5 and Sunrise varieties, than in the S37-388RR plants. It should be added that the extent of infection in S37-388RR roots is much more restricted as well, so that this variety is probably more resistant to P. halstedii than the other two.

It is hard to explain the decrease in infection of CM5 seedlings growing in infested soil stored for increased lengths of time, as shown in Table I, while the infection of the variety Sunrise remained at the same level. Since Tables II and III do not show this decrease, it is unlikely to be significant.

TABLE III

Plants with fungus in the roots,

but no symptoms on the leaves

Age of soil	Age of plants	Sunflower varieties								
		CM5			Sunrise			S37-388RR		
months	weeks	Inf.	Total	%	Inf.	Total	%	Inf.	Total	%
4	6	11	30	37	13	26	50	12	44	27
28	6	6	11	54	5	16	31	7	17	41

VI - SPORE INOCULATION EXPERIMENTS

The preparation of spore suspensions containing zoosporangia from the leaves and cotyledons of infected plants, has already been described in the section on Materials and Methods. The standard inoculation procedure was as follows: four inch pots were filled with pasteurized soil to within 5 cm of the top; five seeds were placed on the surface of the soil, and then 1 cc of spore suspension was placed on each of the seeds, which were then covered with a further 2 cm of soil. If the suspension had been adjusted to the standard density, then 1 cc would contain a total of 150.000 spores.

The pots were kept in a greenhouse where the temperature rarely dropped below 20°C, but often reached 30°C on sunny days. Other experiments were conducted under more closely controlled conditions in controlled environment cabinets, where the temperature would remain constant day and night, and light could be given for a predetermined number of hours at from 600 to 1500 foot-candles measured at plant level.

Experiment (i)

Tests were carried out to determine the effect of inoculation with spore suspension. The results are given in Table IV. The number of infected plants was determined by inducing spore formation.

TABLE IV

Spore inoculation tests

Sunflower variety	Age of plants	Number and percentage of infected plants			Grown in	at
	days	Inf.	Total	%		
CM5	13	50	73	68.5	Greenhouse	21°
Sunrise	13	18	24	75.0	Greenhouse	21°
CM5	14	55	78	70.5	Cabinet	21°
CM5	14	104	109	95.4	Cabinet	21°
CM5	16	74	103	72.0	Cabinet	21°
CM5	16	87	102	86.0	Cabinet	21°
CM5	16	73	79	92.4	Cabinet	21°
CM5	17	88	105	83.8	Cabinet	21°
CM5	19	54	60	91.6	Cabinet	21°
CM5	20	72	73	98.6	Cabinet	21°
CM5	20	60	75	80.0	Cabinet	21°
CM5	23	57	61	93.5	Cabinet	21°
Average		839	992	84.58		

During some of the earlier experiments it was noted that a few of the uninoculated check plants in the cabinets showed signs of infection. Tests were made, and showed that the pots and soil used, were free from contamination. However, plants bearing ripe zoosporangia which were shortly to be used to make spore suspensions, were sometimes present in the cabinets. The moment the plants were removed from the cabinets, there were no further infections of the checks.

Attempts were made to calculate the extent of infection, by estimating the percentage of the lower surface of the cotyledons and the leaves actually covered with the sporangiophores of the fungus. This proved to be of little value, however, as it was found to vary greatly from plant to plant and experiment to experiment, and even from leaf to leaf in a single leaf pair.

Experiment (ii)

In most of the tests damping-off was common, as a result of the Plasmopara inoculation of the seeds. The cotyledons would unfold but not reach their full size, when growth of the seedlings was halted, and after one or two days the seedlings fell over. Infection by Plasmopara was thought to be the cause. However, the possibility that other fungi were involved had to be investigated.

For this purpose plants were inoculated and grown in sterilized soil and pots, for 15 days at 20°C in a controlled environment cabinet. The variety CM5 was used, and the fungus was

induced to sporulate on 94 % of the seedlings (47/50 plants). Ten seedlings showing damping-off symptoms were plated on potato-dextrose-agar. Each seedling was divided into five short lengths, thus forming a total of 50 lengths. Fungal isolations were obtained from 2 of the 10 seedlings, with the following fungi isolated:

Trichoderma viridis (twice)
Pythium debaryanum (twice)
Fusarium sp. (thrice)
Stemphylium sp. (once)

This low number of isolations is taken as an indication that Plasmopara was responsible for the damping-off. Since sterilized soil was used and the seeds had been surface sterilized with formalin before planting, no other fungi had been expected. However, air contamination or presence of fungi within the hull, might account for the isolations that were made.

Experiment (iii)

Except for the previous experiment, only pasteurized soil had been used, but it seemed worth while to compare the effects of using sterilized soil, to see if there would be any difference in the amount of infection. Soil, already pasteurized, was therefore sterilized by autoclaving it in the pots, for 3 hours at 15 lbs. pressure. It was then used the following day. Fifty seeds of the variety CM5 were planted and inoculated in each type of soil, and the seedlings grown for 14 days in a controlled

environment cabinet at 20°C. When formation of spores had been induced, 65 % of the plants in sterilized soil showed signs of infection, as compared to 33 % of the plants in the pasteurized soil. Both these percentages are rather low, compared to the results presented in Table IV. This is probably due to insufficient light being available in the cabinet in which the plants were grown. Low light intensity reduces the percentage of infected plants, as determined by the spore induction method, which will be shown in later experiments. But as the two sets of plants were grown under identical conditions, the results can still be compared.

The lower percentage of infected plants in the pasteurized soil may have been caused by interference of other microorganisms with the Plasmopara, but it is possible that the physical process of autoclaving had induced some changes in the soil that led to this difference.

As the percentage of plants listed as infected, depends on the number in which sporulation can be induced, a small difference in the growth rates of the seedlings in the two types of soil could have led to the difference in the percentage of infection that was found in this experiment. A slightly faster growth of the seedlings in the sterilized soil, can make it much more difficult for the fungus to keep up with the elongation of the stem, so that in many seedlings it may be unable to spread into the cotelydons and the leaves, which then will be listed as free from infection.

Experiment (iv)

The fungus was usually induced to sporulate overnight, while the plants were kept in the dark, but in one experiment seeds were inoculated with spores which had formed in the cabinet with the light left on all night. Seventy-five seeds of the CM5 variety were inoculated, and the seedlings grown at 20°C for 15 days, and after sporulation had been induced, 88.5 % of the plants was found to be infected. Consequently light does not seem to have had any effect on the germination or infectivity of the spores.

Experiment (v)

In these tests CM5 seeds were sown in sand and, when they had germinated, the seedlings were transferred to a nutrient solution in a glass dish, supported by a plastic grid, and held in place by some cotton wool around the hypocotyl. The plants were kept in a cabinet at 21°C, and after 24 hours were inoculated by adding spore suspension to the nutrient solution. Four days later, the seedlings were planted in soil. After 12 days two plants, out of a total of 21, showed heavy symptoms in all the leaves, while another two showed the characteristic symptoms along the veins. Although the plants were observed continuously for another 3 weeks, no further plants showed any symptoms. Therefore, 4 infected plants give a 19 % infection, which is very much lower than in the case of the spore inoculation tests in soil, reported in Table IV. It is true, that in this former test, sporulation was induced in the seedlings before the symptoms developed on the leaves. In most

of the seedlings the infection was so severe, that if sporulation had not been induced after 14 days and the number of plants showing it determined, the infected seedlings would have died before the leaves had developed far enough to show symptoms.

Experiment (vi)

In this experiment seeds were sown in vermiculite, inoculated with spore suspension after five days when the seedlings had emerged, and transplanted to soil a week later. After transplanting, the seedlings were covered with a plastic bag, which was also wrapped around the pot to keep the humidity high enough to give the plants a chance to recover. Next day sporulation was observed on one seedling. Condensation in the bag and on the plant had apparently provided the same spore induction conditions, as were provided by a humidifier running overnight in a cabinet.

One month after the first infection had been observed, another plant developed symptoms, in this case, along the veins of the leaves, giving a total of 13.3 % infection. The plants were kept in the greenhouse and only discarded two months after infection, since no further symptoms developed.

As with the previous experiment, the plants here showed infection more slowly than those inoculated in soil, so that spore induction could not take place when the seedlings were two weeks old. It should also be borne in mind, that the number of seedlings contracting the disease, decreases rapidly with increased age, as described by Yagodkina in 1956.

Experiment (vii)

In still another experiment, seeds were inoculated with spore suspension as they were being planted in vermiculite, following the same procedures as for spore inoculations in soil. The pots were kept in a controlled environment cabinet at 21°C for 8 weeks, and out of a total of 75 plants, only 2 showed any symptoms on their leaves, or 2.7 %. In this case the age of the seedlings can not have played a role. Perhaps the special growing conditions in vermiculite, with a different humidity than in soil, led to this low percentage of infection. The plants of the previous experiment (vi), sown in vermiculite and inoculated after 5 days, and then transplanted into soil after another week, did much better. They reached an infection percentage of 13 %, although the number of plants was much lower (2/15). None of these experiments were repeated, since they only represented investigations of an exploratory character, from which no very definite conclusions were drawn.

Experiment (viii)

In order to compare the success of infection by way of oospore infested field soil (as described in section V), with that of a spore suspension, nine Russian varieties of sunflower were subjected to both methods of inoculation. For each test, 25 seeds of each variety were used, five seeds to a pot. The plants were kept in a cabinet at 20°C.

Seven weeks after seeding, the plants grown in the infested soil did not show any symptoms, so the roots were collected, and examined for infection.

The spore inoculated plants were grown for 16 days, and then induced to sporulate. For this test a group of CM5 seedlings was included to give a basis for comparison with the other experiments. Sporulation on the seedlings was heavy, and on the majority of the cotyledons of the infected plants, the whole lower surface was covered with the spores. The results are given in Table V.

Comparison of the results shows, that spore inoculation is more effective than growing the seedlings in infested soil.

TABLE V

Infection of Russian sunflower varieties

Variety	Spore inoculation test sporulation induced	Infested soil test fungus in root sections
SPV 1	63.5 %	36.4 %
SPV 2	80.0 %	41.6 %
SPV 3	96.5 %	21.7 %
SPV 4	80.0 %	23.8 %
SPV 5	92.0 %	30.5 %
SPV 6	56.5 %	38.1 %
SPV 7	78.3 %	47.8 %
SPV 8	73.9 %	50.0 %
SPV 9	68.8 %	29.1 %
CM5	65.7 %	-
Average(except CM5)	77.1 %	35.7 %

VII - INFLUENCE OF TEMPERATURE AND LIGHT ON INFECTION

Preliminary tests were carried out to determine the influence of light and temperature on the rate of infection of seedlings inoculated with spore suspension. Two temperatures were used, 21°C and 24°C. Pots, with seeds inoculated as described in the previous sections, were placed in controlled environment cabinets and watered daily. Fluorescent lighting was used to give a 14 hour day. After 12 days, sporulation was induced with a humidifier. It was found that infection was higher at 24°C than at 21°C. The results are presented in Table VI.

The plants grown in the 21°C cabinet were taller than those grown in the 24°C cabinet. As 24°C is below the optimum temperature for the growth of sunflowers, the difference in temperature was probably not the factor involved. However, the light in the 21° cabinet proved to be 600 foot-candles compared to 1200 foot-candles in the other one, both at plant level. Plants grown at the same temperature, and given these two different amounts of light, show a marked difference in growth. Below 1000 foot-candles at plant level, the seedlings become etiolated, and the hypocotyl is 10 cm or more long, before the first leaves start to develop. At light intensities over 1000 foot-candles the leaves develop when the hypocotyl is only 5-8 cm long.

This difference in height has a very important influence on the number of seedlings showing sporulation of the fungus. After emergence, the hypocotyls of the etiolated seedling elongate so quickly, that the fungus has to grow much faster than in the non etiolated seedlings to keep up with the growing point. It has been observed that once the fungus lags behind, it can rarely overtake the meristem, at least in the relatively short term experiments conducted here.

In order to separate the influence of the two factors, therefore, another experiment was set up. This one started the same way as the previous test, but after the emergence of the seedlings, half of the pots of each set were transferred to the other environment. As the influence of the light had not had time to affect the seedlings, the influence of temperature during emergence might become clear later, when sporulation would be induced. The plants were divided into four groups. Group 1 was kept at 21°C for 14 days; group 2 was kept for 4 days at 21°C, and then for 10 days at 24°C. Group 3 was kept at 24°C for 14 days, and group 4 for 4 days, while it was kept at 21°C for the next 10 days. The results were determined by induction of sporulation and are given in Table VII.

Infection was higher in group 2 and 3, which had been grown at 24°C and 1200 foot-candles, except for the first 4 days in the case of group 2. This could be ascribed to the influence of light, in part at least. But the infection in group 4 was higher than in group 1, and in 3 higher than in 2. From this, a higher

TABLE VI

Influence of temperature on infection

Temperature	Number of plants		
	Infected	Total	Percentage
21°C	18	42	43
24°C	31	49	63

TABLE VII

Influence of temperature on infection

Temperature		Number of plants		
		Infected	Total	Percentage
Group 1	21°C for 14 days	19	45	30.8
Group 2	21°C for 4 days 24°C for 10 days	23	48	46.6
Group 3	24°C for 14 days	35	48	72.8
Group 4	24°C for 4 days 21°C for 10 days	20	35	57.0

infection at 24°C than at 21°C might be deduced, at least during the first 4 days after infection, as the plants were in the 24°C cabinet during that time.

VIII - INFLUENCE OF THE CONCENTRATION OF SPORE SUSPENSIONS ON INFECTION

A series of tests were carried out in order to find how spore concentration of the suspension, influences the percentage of plants infected.

For this purpose, the standard concentration of 150.000 spores per cc was used at first, but later a concentration of 100.000 per cc was used, as it facilitated the calculation of the spore numbers in the greater dilutions. For all tests 50 seeds were used, five per pot, for each concentration.

In the first test, the standard 150.000 spores per cc of solution was diluted by adding an equal quantity of tap water. The next dilution was made by taking half of the previous dilution and adding an equal quantity of tap water. This procedure was repeated several times, in the same way, to give all the dilutions used for inoculation as listed in Table VIII.

From the results of test a and b of Table VIII, it was clear that the limit of dilution, which will still result in infection, had not yet been reached. Therefore, the dilution range was expanded in the next experiment, with the results as presented in Table IX.

TABLE VIII

Influence of spore concentration on infection

Test a

Spores per cc	Plants infected
150.000	67 %
75.000	87 %
37.500	70 %
18.750	73 %

Test b

Spores per cc	Plants infected
150.000	83 %
30.000	81 %
7.500	60 %
3.000	70 %

TABLE IX

Influence of spore concentration on infection

Spores per cc	Plants infected
100,000	95.8 %
20.000	98.0 %
10.000	95.5 %
2.000	78.1 %
1.000	95.5 %
500	57.5 %
100	62.0 %
Check	0 %

From these results, it was seen that inoculation with 1000 spores per cc per seed still gave a very high rate of infection, in fact practically as high as the maximum concentration of 100.000 spores per cc. The infection percentages obtained by inoculation with 500 and 100 spores per seed, are lower. This probably indicates that here the number of spores becomes the limiting factor. The differences seen in the percentage of infection in sets of plants inoculated with more than 1000 spores per seed, can probably be ascribed to variability inherent in the materials studied, or "error of experiment".

IX - INFLUENCE OF STORAGE CONDITIONS ON VIABILITY OF SPORES

Since it was impracticable to maintain sporulating cultures of the pathogen on plants at all times, several methods of preservation were tested, in an attempt to have viable and virulent spores available at short notice, as and when needed.

As a first test, the spores were simply left on the plants, which were kept at 20°C in the cabinet where the fungus had been induced to sporulate. For a period of 1 week, a fresh spore suspension was prepared from this material on certain days, and used for inoculations. Part of the suspension made on the first day was stored at room temperature and used for inoculations on the second and third day. After 3 days it was discarded since large numbers of bacteria had developed. The number of infected plants was determined by induction of sporulation (Table X).

From the results presented in Table X, it appears that there is very little loss of infectivity during the first 7 days after spore induction under these conditions. Since the standard concentration of 150.000 spores per cc was used, the results may not give a true picture of the loss of infectivity, however, since other tests have already shown that 1000 spores per cc are as effective in infecting, and small numbers may have survived.

In a second test, spores still on the leaves were kept in petri-plates at room temperature, and either in a refrigerator

TABLE X

Viability of spores stored at room temperature

Age of spores	Infected plants (percentage)	
Days	Fresh suspension	Stored suspension
1	96.5	-
2	96.4	57.1
3	96.1	60.7
4	80.0	-
6	70.3	-
8	80.2	-

or in a freezer. At different intervals suspensions were prepared and used for inoculation, 50 seeds being tested in each case. The number of infected plants was determined by induction of sporulation. As the results, presented in Table XI, show, storage in a freezer was so successful, that it became the standard method of keeping inoculum available.

In a third test on this subject, an attempt was made to find how long the spores could survive in the freezer. Equal amounts of leaf material that had been stored for different periods of time, were used in the preparation of the spore suspensions. The seedlings were grown for 15 days at 20°C, and 50 seeds were inoculated with each. The number of infected plants was determined by induction of sporulation (Table XII).

The results, as presented in Table XII, indicate that for practical purposes, the limit of time during which spores can be stored for use in inoculation experiments, is from 3 to 4 months.

TABLE XI

Spores stored under different conditions of temperature

Age of spores	Percentage infection		
weeks	freezer (-20°C)	refrigerator (4°C)	room temp.
1	-	87	0
2	-	2	0
5	76	2	0

TABLE XII

Viability of spores stored in a freezer

Age of spores	Percentage infection
3½ months	10
6 months	0
9 months	0

X - INFESTATION OF SOIL AFTER A SPORE INOCULATION TEST

Soil in which infected plants have grown, becomes infested with P. halstedii. Subsequent sunflower plants grown in this soil may become infected. The experiments described in this section were designed, in order to find out what the source of this infectivity is, and how soon after spore inoculation of seedlings this long term infectivity can occur in the soil.

There seem to be two possible sources for this infectivity. First, the zoosporangia, produced primarily on the leaves of the plants, which can fall to the ground and be washed down into the soil. Secondly the oospores, which are formed in the roots and the lower stem. The nature of these two possible means of propagation seems to make it less likely that the zoosporangia will be responsible for the infestation of the soil, since they are thin walled and can hardly be expected to survive in it very long. On the other hand oospores with thick resistant walls, could very well be present when the soil is tested after longer periods of time. All this, however, had to be tested.

When seeds were planted in soil, which had been used for 2 weeks in a spore inoculation test, the seedlings became infected. This could, of course, have resulted from either zoosporangia from leaves and cotyledons of the first test, or from oospores, formed in the roots of the plants which had deteriorated so far that they came off, when the main root was pulled out. So a number of tests

were devised in order to find the right explanation.

In the first of these tests, soil from several previous spore inoculation tests was re-planted immediately after the end of spore inoculation tests (which had lasted 2 weeks). The age of the soil, as given in Table XIII, refers to the lapse of time since the end of the spore inoculation test. The new seedlings were grown in a controlled environment cabinet at 20°C, and the number of infected plants was determined by induction of sporulation. The results are presented in Table XIII.

The level of infection was as high as in the case of the spore inoculations. To try to find out if the infectivity might still be caused by spores surviving from the first spore inoculation, tests, the same soil was used for a third time. The conditions were the same as in the previous experiment, except for the lapse of time since the original spore inoculation. Three separate tests gave the results shown in Table XIV.

This level of infection is almost as high as that of tests, where the soil had only been re-planted once, and would appear to make the theory of infection by surviving spores from the spore inoculation test less likely. However, several of the seedlings were deformed and stunted, some with hardly any leaves, and others with abnormal ones. As the sporulation of Plasmopara was light on most of these seedlings, and the symptoms were very different from those observed on infected plants in other experiments, it was thought that metabolites and decomposition

TABLE XIII

Percentage infection of plants in re-planted soil

Number of plants		
Infected	Total	Percentage
150	167	90.0
142	157	90.4

TABLE XIV

Percentage infection of plants in soil in second replanting

Number of plants		
Infected	Total	Percentage
29	33	87.8
77	117	65.8
108	127	85.0

products from the sunflowers grown previously in this soil, were the cause of these abnormalities. This phenomenon made it difficult to use the soil more than once, and thus it was not possible to check how the infectivity of the soil would develop, when plants were grown in it repeatedly.

In order to find out how long the soil would remain infective after being used in a spore inoculation test, a certain amount of it was collected and stored at room temperature, the empty pots being kept under the same conditions in case they too had been infested. At regular intervals, 5 seeds were placed in each of 10 pots, and the plants were grown in a cabinet at 20°C. After approximately 2 weeks growth, the number of infected plants was determined by the sporulation induction method (Table XV).

The surprisingly high infection at 8 and 13 weeks, after the continued decrease in the earlier tests, can probably be explained by the dryness of the soil used. Particularly in the tests at 5 and 7 weeks, the soil had become very dry, and it proved very difficult to water the pots, as water would stay on the surface of the soil and had to be worked in with pot labels. The seeds were slow to germinate, and emergence was spread over a long period of time. The induction of spores might therefore have come at a time, when fewer seedlings would be showing the spores of the fungus than were actually infected. In the case of the tests after 8 weeks and after 13 weeks, the soil was carefully moistened and mixed before it was put in the pots.

A humidifier was running in the cabinet where the 13 week series of plants was being grown, and it could be seen, that there were more symptoms in the leaves than usual in this type of experiment. No water had been present on the surface of the leaves, and there had been no attempt to induce sporulation. The latter was achieved, however, by running a humidifier of the same capacity in the much smaller cabinet where the spore induction usually took place.

The results of this experiment made the idea seem highly improbable, that the infection arose from surviving zoosporangia, but it still did not prove whether zoosporangia or oospores were causing the infection. As a further test, to see how long zoosporangia would stay infective in the soil, a spore suspension was prepared and poured onto the surface of the soil in the pots, in the same way as the usual spore inoculations were carried out. Another 2 cm of soil was then added. Soil treated three different ways was used, sterilized, pasteurized, and unpasteurized. The check was unpasteurized and uninoculated soil. For the first test, seeds were planted immediately after the preparation of the pots. They were pushed down with the aid of a pencil. The infection was checked by induction of sporulation. The results are given in Table XVI.

TABLE XV

Percentage infection of plants in re-planted soil at varying intervals after collection

Time after collection	Number of plants		
Weeks	Infected	Total	Percentage
0	22	38	57.9
1	17	49	36.0
2	9	50	18.0
5	5	47	10.6
7	4	42	9.5
8	20	55	36.4
13	35	54	64.8

TABLE XVI

Survival of zoosporangia in soil

Seeds planted after	Percentage infection		
Days	Sterile soil	Pasteurized soil	Unpasteurized soil
0	94[1])	100	61
3	79	82	48
7	36	62	56
14	4	8	0
28	0	0	0

[1]) The check plants were free from infection in all tests.

XI - WORK ON WILD SUNFLOWER SPECIES

During the summer of 1964 severe infection by P.halstedii was discovered in a field plot of the wild sunflower species Helianthus tuberosus at Macdonald College. The plants had come from Manitoba three years before, and may have carried the infection in their rootstocks, although no symptoms had been seen in previous years. However, for two years a plot of infected Sunrise plants had been present some 50 m away, so that spores might conceivably have been blown across from these plants.

The symptoms of the infection in H. tuberosus were somewhat different from those seen in H. annuus, as no yellow areas developed on the leaves. However, stunting did occur, the internodes between the leaves being much shorter than those of apparently healthy plants in the same plot. The visibly infected shoots were from 15 to 25 cm high, while those showing no symptoms were approximately 50 cm tall(Figure 10). In the diseased plants the leaves were much narrower and smaller, their lower surface covered by the sporangiophores of the fungus. The leaves of the infected plants looked somewhat darker green, and thicker, more like the older leaves of the healthy plants. However, some had dried out, and growth of the diseased plants seemed to have stopped altogether.

Some infection was also found in another plot of H. tuberosus, but none in any of the plots of the other wild species.

H. maximiliani, H. laetifolius, and H. subtuberosus.

It seemed worth investigating whether there were differences between the fungus on the H. tuberosus plants and the eastern Canadian isolate. Infected leaves were collected and a spore suspension was prepared. Suspensions were also prepared from spores from CM5 leaves in the freezer, and spores from Sunrise plants in the field, and were used to inoculate seeds of the variety CM5. After inoculation the plants were grown at 21°C. After 18 days the number of infected plants was determined by inducing spore formation. The spores of each isolate were collected and used for another inoculation. This procedure was repeated again, in order to see if the second and third generation of the isolate from H. tuberosus would become more virulent to H. annuus than the first, after passage through it.

From the results given in Table XVII, there does not seem to be a difference between the three types of inoculum. The variation in infection percentages might have been just as large if three groups of plants had been inoculated with the same spore suspension. There is no indication that the isolate from H. tuberosus is less virulent on H. annuus than the other two isolates, or of adaptation to H. annuus after passage through it.

TABLE XVII

Infectivity of different isolates

Isolate from	Generation 1	Generation 2	Generation 3
	percent	percent	percent
H. annuus			
spores from freezer	50	45	69
spores from field plot	83	62	82
H. tuberosus			
spores from field plot	79	30	55

Having found the isolate from H. tuberosus able to attack H. annuus, the reverse inoculation was then carried out. Young shoots of the four wild sunflower species, H. tuberosus, H. maximiliani, H. laetifolius, and H. subtuberosus, were collected in the field plots and planted in pots in a greenhouse. They were inoculated by addition of spore suspensions to the soil in which they were planted. Those plants that survived, were collected after four months, the shoots being cut off at soil level, as the roots were found to have developed side shoots that had not yet emerged, which might be required for further tests.

The harvested shoots had not shown any symptoms, so they were sectioned to see if the fungus was present in the lower part of the stem. In the two shoots of H. subtuberosus nothing was found, in one shoot of H. maximiliani some intercellular mycelium was present, that may have been Plasmopara, while in all three H. laetifolius plants tested some mycelium of the fungus was found. A larger amount of mycelium was present in sections of the only H. tuberosus shoot available, which decreased from the lower part of the hypocotyl to the node where the first leaves arose. However, this infection could have been present when the shoot was collected in the field, as it was in the plants of this species that the fungus had been found. After another two months, a further side shoot of H. laetifolius was found to have intercellular mycelium with a few haustoria.

In a further test, seeds of the four wild species were sown in the usual method in pasteurized soil, and the seedlings inoculated when they were transplanted after emergence. The reason for the deviation from the normal inoculation method, was the very irregular and slow germination of the seeds of the wild sunflower species used. Of each of the four species, several lots of seeds, harvested in different years and in different locations, were planted. In several samples none of the seeds germinated, while in others the germination was very poor. It was believed that a better comparison of the relative susceptibility of the four species could be made if all the seedlings were inoculated at the same time, although it is true that they would probably be of different sizes. With the procedure followed it was at least certain that the seeds which did not germinate had not succumbed to Plasmopara. No symptoms were seen after three months and the plants were discarded.

Figure 10. Helianthus tuberosus, infected by Plasmopara halstedii

XII - SECONDARY INFECTIONS

In the P. halstedii literature, invasion of the root is called primary infection, while the term secondary infection is used for penetration of the leaves of healthy plants by zoospores. The possibility of secondary infections had been considered more than 80 years ago by Farlow (1883), who thought it highly unlikely that the zoospores of P. halstedii could penetrate the uninjured upper surfaces of large sunflower leaves. Young and Morris (1927) reported that all the inoculations of the upper surfaces of such leaves, which they made in the field, failed to result in infections. However, Yagodkina (1955) reported local infections on leaves. Therefore leaf inoculation tests were carried out to try to repeat her work.

To this end seedlings of various ages were inoculated by putting drops of spore suspension on the leaves. Some leaves were untreated, in others the leaf surface was first damaged by rubbing it either with or without carborundum powder, by scraping it with a razor blade or by using sandpaper, the last proving to be the most satisfactory method. After inoculation the plants were covered with a plastic bag to keep the humidity at a high level, as this was expected to be imperative for successful infection by zoospores. Some plants were placed in a cabinet at 20°C, but most were kept in the greenhouse.

The results of several of the tests were negative, but in a few there was definite evidence of secondary infection. In a series of seedlings inoculated when they were two weeks old, three small spots of 2-3 mm diameter were found on the leaves 1 week later. When sections were made, intercellular mycelium was present, but no haustoria were seen.

In another test with 2 week old seedlings, more spots of the same type were seen 4 days after inoculation. Two days later small patches of a thin layer of sporulation of Plasmopara had developed on the upper surface and the number of spots had increased, with some leaves having up to 10 spots. When sections were made, the fungus was found to be present in the petioles and also in some of the stems, even down to below the soil level. When sporulation was induced, 23 % of the plants produced zoosporangia. As check plants, which had been injured, covered with a plastic bag and grown under the same conditions as the inoculated plants, did not show sporulation of the fungus; the results seem to indicate that secondary infections by Plasmopara are possible, and that they can lead to infection of the rest of the plant as well. This is similar to the situation described by Nishimura (1922) in Helianthus divaricatus, where infection was said to take place through the stomata, and the fungus subsequently progressed down the stem to the rhizome.

XIII - GRAFTING EXPERIMENTS

Results of the spore inoculation experiments seemed to indicate that seedlings had to be inoculated while very young if the infection was to spread upwards, otherwise only the roots would become infected. This could be due to an increase in resistance of the plants with age, perhaps localized in the stem.

In order to test this point, it was decided to make graftings of healthy and diseased plants. Seeds were inoculated in one tray, while healthy seedlings were grown in another. Later they were transplanted two by two into pots and after one or more days the actual grafting took place. Cleft-grafts were performed and the graft surrounded with moist cotton to make it take. After a week the cotton was removed, and a few days later the stem of the diseased seedling was cut.

The gratings were essentially done one way, i.e. the shoot of the diseased plants grafted onto the stem of the healthy ones, as the roots of the infected plants were often already too severely infected to support their own stems. In some cases the shoot of the healthy plant was removed, generally it was left in place. Several successful grafts were obtained with healthy stocks, and scions which clearly showed symptoms in the leaves. They were left in the greenhouse for up to 5 weeks in this state.

Even after this time none of them showed any symptom of Plasmopara infection in the leaves of the stocks. Therefore, these plants were collected, and sectioned at the place where the graft had been made. In one out of 10 plants the fungus was found to have spread a maximum of 3 mm into the healthy plant; in all others it was only present in tissues originating from the diseased seedling. This observation then, might indicate that there is some form of resistance in the stems of older plants, which seems to be lacking in very young seedlings.

It had been observed in other tests that some seedlings growing either in infested field soil or inoculated with spores, had the first symptoms appear in the younger leaves, bypassing many lower ones. This happened in plants of approximately the same age as those discussed in this section. This can also be observed in plants growing in the field. Perhaps, therefore, some factor is at work that only operates in grafted plants.

XIV - GERMINATION OF ZOOSPORANGIA

Experiments were carried out to test the rate of germination of zoosporangia. At first distilled and tap water was used, but this gave a low rate of germination, the maximum being 10 %, and the zoospores that were formed disintegrated shortly after emergence from the zoosporangia.

This led to the idea that the osmotic pressure of the solution might be important, but tests in saline solution (0.9 % NaCl) gave negative results. However, when sucrose solutions were tested, the germination reached 50 to 70 %, and the zoospores developed further, i.e. after being motile for a few hours they became rounded off with a heavier wall and a germ tube was formed.

In subsequent tests three different temperatures were tried: 12°, 15°, and 18°C, while the concentration of sucrose varied from 0.25 % to 4 %. Germination was highest, and about equally fast, at 15° and 18°C. Tests with sucrose at 0.25 % showed very little difference with the water check, but a 0.5 % gave up to 25 % germination, while 1 and 2 % were equally good, giving from 25 to 60 % germination. The 4 % sucrose solution sometimes gave 60 % germination, but at other times practically none.

When germination was high, the other stages developed as well. A typical time sequence for a test at 15° or 18°C would

be: a maximum germination of 50 % reached after 4 hours, when the first zoospores would cease to be motile and develop a thick wall. The first germ tubes appeared after 5 hours, and after another 2 hours they were up to ten times the length of the zoospore. Spores that germinated rapidly, also went through the subsequent stages rapidly. Thus when the first zoospores had completed the formation of their germ tubes, a few others might still be motile.

In a few tests at 20° or 23°C direct germination of zoosporangia by way of germ tubes was observed, but never more than one or two spores amongst several thousand.

XV - WORK ON THE PROBLEM OF STUNTING OF INFECTED PLANTS

Extracts of healthy and infected plants were tested for their content of growth substances, primarily indolacetic acid (IAA), by means of the Avena coleoptile straight growth test and by paper chromatography.

The extracts were prepared according to procedures described by Srivastava, Shaw and Vanterpool (1962), except for a few minor changes. First, the plant material was frozen in a freezer after collection, instead of the Dry Ice used by Srivastava et al.; then it was extracted for 24 hours in absolute alcohol in a freezer, and finally the alcohol was evaporated at 35°C and the residue redissolved in a buffer solution. The buffer solution was a mixture of 99 parts M/100 potassium dihydrogen phosphate and 1 part M/100 disodium hydrogen phosphate. The pH has an important influence on the results, and this mixture had been reported to have the optimum pH for these tests (Larsen 1955). After filtration, the extracts were used in the straight growth coleoptile test. For the paper chromatograms, the pH was adjusted to pH 3 by adding hydrochloric acid, and the extract was shaken with ether. The ether fraction was then applied to the paper.

(a) Avena coleoptile straight growth tests.

Seeds of the hull-less variety "Brighton" of Avena sativa, obtained from the Canada Department of Agriculture Research Station in Ottawa, were used to grow the coleoptiles

required for the straight growth tests, which were conducted according to methods described by Larsen (1955). The seeds were first soaked for 2 hours, then planted in moist vermiculite, and kept in the dark. For the last 12 hours before the coleoptiles were to be used, they were illuminated with red light. They were cut and handled in the same red light, with the top 3 mm being discarded, and the next 4 mm being cut off as the section to be used. The coleoptile sections were measured by projecting their image on a sheet of paper on a table, with an accuracy of 1 %.

Before the tests were made with the various plant extracts, a series of dilutions of pure IAA in buffer was used, to calibrate the method and to see what range of variation might be expected (Table XVIII). The results (Table XVIII) agreed with those reported by Larsen (1955).

In the first test in which plant extracts were used, they were prepared from old frozen leaves, fresh leaves ground up in a mortar, and from fresh leaves cut into small pieces, respectively. Each test was repeated; both sets of results are presented. The results for a series of concentrations of pure IAA solutions are included (Table XIX). They show inhibition by the plant extracts in most cases, when compared with the buffer check. Although there is a large discrepancy between the results obtained from the replicated extracts, the highest amount of growth stimulating substances seems to have been present in the extract of ground tissues.

TABLE XVIII

Elongation of *Avena* coleoptile sections in solutions with different IAA concentrations

Solutions used	Elongation		
	in mm	in percentage	% of check
Buffer check			
Mean	0.62	15.6	100
Range	0.60 - 0.68	15.0 - 17.0	97 - 109
IAA 25 µg/l			
Mean	0.73	18.3	122
Range	0.60 - 0.92	15.0 - 23.0	100 - 153
IAA 50 µg/l			
Mean	0.86	21.5	143
Range	0.79 - 1.00	19.8 - 25.0	132 - 167
IAA 75 µg/l			
Mean	0.95	23.8	158
Range	0.88 - 1.09	22.0 - 27.3	147 - 182
IAA 100 µg/l			
Mean	0.99	24.8	165
Range	0.81 - 1.10	20.3 - 27.5	135 - 183

TABLE XIX

Elongation of Avena coleoptile sections in extracts of healthy sunflower seedling leaves

Plant extracts	Growth in per cent of buffer check			
	Dilutions			
	1x	2x	5x	10x
Fresh leaf pieces				
test 1	29[1])	33	56	66
test 2	54	81	92	106
Fresh ground tissue				
test 1	-	110	108	94
test 2	89	119	98	67
Old frozen leaves				
test 1	46	56	63	110
test 2	35	44	125	104
IAA solutions check	Concentrations			
	100 µg/l	75 µg/l	50 µg/l	25 µg/l
	183	150	169	166

[1]) Mean growth of buffer check sections was 0.48 mm or 12 %.

In the next tests diseased and healthy plant tissues were used. In every case 10 sections were placed in each vial of diluted extract. The results are presented in Table XX. In two tests there was more growth of coleoptile sections in extracts of diseased than of healthy tissue; in one there was more elongation in extract of healthy than of diseased tissue; in one there was no consistent difference between the two.

(b) Paper chromatograms.

The chromatograms were run with an isopropanol-ammonia-water solvent for 9-12 hours, and sprayed with a ferric chloride and perchloric acid mixture. Three extracts were tested, and in only one extract of healthy tissue a very light patch of colour developed. Since these tests proved unsuccessful, no further paper chromatograms were made.

TABLE XX

Elongation of Avena coleoptile sections in extracts of diseased and healthy sunflower seedling leaf tissue

Plant extracts	Growth in per cent of buffer check		
	Dilutions		
	1x	5x	10x
Test 1			
Diseased plants	128[1])	123	147
Healthy plants	156	173	156
Test 2			
Diseased plants	134	141	142
Healthy plants	120	130	141
Test 3			
Diseased plants	100	119	108
Healthy plants	88	103	92
Test 4			
Diseased plants	96	109	129
Healthy plants	87	117	120
IAA solutions check	Concentrations		
	100 µg/l	25 µg/l	10 µg/l
Test 2 [1])	186	134	94
Test 3	174	142	119
Test 4	182	140	142

[1]) Mean growth of buffer check sections for test 1 was 0.57 mm, or 14.3 %; for test 2, 0.64 mm, or 16 %; for test 3, 0.73 mm, or 18 %; and for test 4, 0.45 mm, or 11 %.

[2]) No IAA solution checks were included in test 1.

DISCUSSION

The first aim of the present work, was to find an inoculation method giving consistently high levels of infection. The spore inoculation methods developed in this work, show that spore inoculation results in a much higher percentage of infected plants, than the method of growing plants in infested soil. As usually over 90 % of the seedlings that were inoculated with spores became infected, as judged by the number on which spore formation could be induced, and as the infection rate remained close to this figure in most of this work, it seems hardly necessary to look for any other method. In some experiments, the only difficulty might be to limit the degree of infection, so as not to lose all the seedlings at too early a stage. Use of low light intensities and spore suspensions of a very low concentration would be helpful, as they were found to decrease the percentage of infected plants.

When the results of the use of infested field soil are compared with those of re-used soil after spore inoculation tests, it is clear that the infectivity of the latter was higher. In the field soil, only a few plants showed symptoms, and after a much longer period of time than in the case of the plants in soil from spore inoculation experiments. Whether the latter soil was used immediately, or three months after the spore inoculation test, did not seriously influence the results. The numbers of oospores in

the field soil may have been much lower than in the re-used soil tested. The fact that some of the field soil samples had been stored under dry conditions for two years,did not appear to have changed their infectivity, when compared with soil collected in the field a few weeks before it was used. Perhaps these spores that had overwintered in the soil in the field for one or more years before it was brought into the laboratory, had to be stimulated into germination by root excretions. Yet, when this soil was reseeded after seedlings had grown in it, the infection in the second series of plants was not any higher than it had been in the first. Therefore, the difference in infectivity of the two soil types, can probably be attributed to the number of oospores present in the respective soil samples.

The fact that oospores were found in the roots of sunflower seedlings within two weeks after seeding and inoculation, is of practical significance. Destruction of infested seedlings has been recommanded as a way of combatting the spread of P. halstedii (Yagodkina 1955). However, by the time the symptoms appear, oospores will already have formed in the roots of the plants. Thus, in spite of the roguing of diseased seedlings, the soil may still become infested, since the underground portions of the infected seedlings, in which the oospores are present, have deteriorated to the point where they can not be collected and destroyed.

Young, Jellison and Morris (1929) reported that severely infected plants lived only for a few weeks and that the survivors failed to produce any viable seed. In the present work, seeds collected from severely infected plants in a field plot not only germinated, but gave rise to normal and healthy plants. Thus no evidence for seed transmission of the disease was found, as has also been reported by other workers (Nishimura 1922, and Spekar 1952). Novatelnova (1963) has shown that seed transmission can occur, but it would appear that seed transmission is not common as a method of spreading the disease. Nevertheless, care has to be taken when importing seed to regions where the disease is absent. Leppik (1962) suggested that sunflowers grown from imported seed be kept under surveillance for the first two growing seasons, and that infected plants could thus be eradicated before the pathogen can pass into the developing seeds. But in the present work it was found that oospores may have formed in the roots of two week old seedlings, and consequently the probability of infestation of the soil when growing imported seed, has to be taken into consideration.

Young and Morris (1927) reported failure of all their inoculations of uninjured upper surfaces of sunflower leaves in the field. In the present work, inoculations of the wounded upper surface resulted in secondary infections. The fact that the leaves were wounded must have been important, as all inoculations on uninjured seedlings failed. The tests were made on seedlings grown

in the greenhouse, where plants are more delicate than those grown in the field. This fact may partly account for the success of the inoculations.

The plants were covered with plastic bags for several days after inoculation, and were wet all that time because of condensation within the bags. As Plasmopara only sporulates when a film of water is present on the surface of the leaves, secondary infection might be expected in the field only where moisture conditions are favorable. Such conditions apparently occur only rarely in nature.

Secondary infections of plants in the field have been described by Yagodkina (1955). From the results given in the present work, it might be concluded that they would probably occur after several rainy days, or when dew is heavy and a film of water can remain on the leaves for prolonged periods of time. Perhaps it is not essential for the host to be continuously wet, as is true for Plasmopara viticola attacking grapes, which can infect in intermittent dry and wet periods, adding up to a total number of hours in which the humidity is over a certain level.

Sporulation of Plasmopara was induced on leaves that had been subjected to secondary infection, and when sections of the rest of the plant were made, the fungus was found to have penetrated into the lower stem and the main root. This would indicate that infection via the leaves, and subsequent spread to the roots, as described for H. divaricatus by Nishimura (1922)

might also occur in the commercially grown varieties of H. annuus, thus leading to a possible infestation of the soil in fields where the disease had not occurred previously.

In all cases where an infected scion was grafted to an uninfected stock plant, hardly any spread of the fungus into the stock plants was observed. The maximum recorded distance was 3 mm, and this was only found in one out of 10 cases. The question is, why does the fungus not penetrate into the stock plant? The tissues of the scion and those of the stock plant must have been in intimate contact, since the graft was able to survive. For Plasmopara the cortex is the most important area of the plant, as it occurs there abundantly, though it is also common in the pith, but it is seldom encountered in the vascular tissuesl Therefore, lack of contact between equivalent tissues does not appear to be the explanation. It may be important that the transport of water and nutrients is almost exclusively one way, into the grafted scions. In the case of secondary infections on leaves, the fungus did spread downwards through the stem into the roots, against the flow of water, but with that of the sugars. The infected scion, being filled with mycelium of the fungus, will probably not be in a position to produce spare sugar that could be transported down into the stock plant. The resistance to penetration into the stems of older seedlings may be related to this situation.

The experiments on the influence of P. halstedii infection on the amounts of growth stimulating substances in sunflower seedlings, described in the present work, were not conclusive. Only a few tests were made, using plant material grown in the greenhouse, and only the Avena coleoptile straight growth test was employed, since work with paper chromatograms proved unsuccessful. To elucidate the problem of a possible hormone imbalance, many more tests would have to be made. It would be preferable to use field grown plants, and more extensive use should be made of paper chromatography.

It is quite possible, that in the stunting of infected sunflower plants, substances with a growth stimulating effect similar to that of IAA are not involved, but some other substances such as gibberellins. In that case it would not be surprising that no clear indications were obtained from the Avena coleoptile straight growth tests. It is also possible that infection has an influence on the growth inhibitors. Since the growth stimulating effect of the extracts seemed to increase with greater dilution, growth in the undiluted and only slightly diluted extracts was often less than it was in the more strongly diluted extracts and the checks. The presence of growth inhibitors may therefore offer an explanation for this phenomenon.

XVII - SUMMARY

The first aim of the present work was to develop a reliable method for inoculating sunflowers with Plasmopara halstedii. The use of a suspension of zoosporangia to inoculate seeds gave consistently high infection. It permitted rapid determination of varietal reactions in 2 weeks, and could be used to determine the influence of various environmental factors on infection.

Normal greenhouse temperatures of from 18° to 25°C, gave good results, and the occasional higher temperatures during the daytime did not interfere. However, the intensity of light was found to have an important influence. It has to be at least 1000 foot-candles at plant level, otherwise the seedlings elongate too fast and the percentage of the seedlings showing infection will be considerably reduced. When several varieties are tested, therefore, care has to be taken to give them equal amounts of light.

A spore suspension containing 1000 spores per cc was found to be adequate for the tests, when 1 cc was applied per seed. The spores that are to be used for these experiments, can be stored on leaves in petri-dishes in a freezer for at least a month after their formation.

The induction of sporulation described above, is a very simple way of determining the percentage of the seedlings in which

infection has taken place and the fungus has spread into the upper part of the plant. The laborious process of sectioning all the roots, to see if the fungus is present, is thus avoided.

Plasmopara halstedii on Helianthus tuberosus in a nursery plot proved to be equally virulent on the H. annuus variety CM5 as the isolate on H. annuus with which most of the experiments were done. According to Novatelnova's (1962) classification of the P. halstedii complex, the isolate from H. tuberosus would be called f. patrus as it infects both perennial and annual Helianthus species.

It has been suggested that the mildew fungus causing severe losses of sunflowers in Russia is a new and more virulent strain (Yagodkina 1955). Nine Russian commercial sunflower varieties that were tested for their resistance to the eastern Canadian isolate were found to be uniformly very susceptible. This makes the presence of a new virulent strain in Russia seem less likely.

No evidence for seed transmission of the disease was found in the present work, although the fungus was seen in the integuments and the embryo of developing seeds in the same series of plants from which the seeds that later were to be tested were collected. The fungus can therefore be presumed to have been present in the embryo of at least some of the seeds that were tested for transmission.

From the work on the infectivity of soil after it has

been used in a spore inoculation experiment, it was concluded that oospores can be formed in very young infected seedlings. Oospores were found in sections of roots of 14 days old seedlings. The weeding out of plants that show infection in plots of sunflowers, which has been recommended as a method to prevent the soil from being infested by the fungus in the USSR, is therefore of doubtful value.

The problem of the cause of stunting of infected plants was investigated by testing extracts of diseased and healthy plants for their content of growth stimulating hormones. In some of the tests there seemed to be more activity in the healthy, in others in the diseased plants. It is therefore very hard to draw any conclusions from the limited evidence available on this point.

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