TEMPERATURE RELATIONS OF SOME MINT LEAF SPOT DISEASES

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TEMPERATURE RELATIONS OF SOME MINT LEAF SPOT DISEASES

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INTRODUCTION

The oil of the various cultivated species of mints is contained primarily in the leaves of the plants. Any disease affecting the leaves may therefore reduce the yield of oil distilled from the herb. There are several leaf diseases of mints which cause necrotic areas on the leaves and partial defoliation. Under certain conditions they may be so severe as to result in serious reduction in oil yield. It is known that in some years the diseases may be very serious and cause extensive defoliation, while in other years there is practically no leaf spot. Such differences are usually associated with variations in the weather conditions from year to year. Temperature and moisture are the most important factors influencing the incidence of leaf diseases. The purpose of this problem was to isolate the pathogenic organisms from several of the leaf diseases, grow them in pure culture, and to study the effect of temperature and moisture on them and the diseases they cause.

THE HOST (2)

There are two main areas under mint cultivation in the United States. One area is located in southern Michigan and northern Indiana, and the other in Oregon and Washington. Small plantings are cultivated in northern Ohio and introductions have recently been made into Wisconsin and Canada. In the midwestern region, acreage under cultivation and yields have decreased steadily in recent years due to inroads made by the mint flea beetle, Verticillium wilt, and possibly leaf diseases. In the Pacific region production has increased markedly in the last 10 years with acreage and yield now ahead of Michigan and Indiana.

New mint plantings are made in rows by the use of rhizomes. Seed cannot be used due to the near sterility of most of the species. After the first year, the rows are obliterated by the spread of the rhizomes and runners and the mint is referred to as "meadow mint." It is in this mint, with its dense, uninterrupted foliage, that leaf spot infections are most severe. Lack of aeration, resulting in a high relative humidity provides excellent conditions for the development of leaf spot. The fact that the mint is grown on the same land year after year provides a ready supply of the inoculum which is carried over from year to year on the fallen leaves.

Mint is ideally harvested when flowering, for this is usually the time when the quality of oil is best.

However, it is often necessary to harvest mint prematurely to prevent excessive defoliation from rust and leaf spot.

The three cultivated mints, American spearmint, Scotch spearmint, and peppermint are all affected by leaf spot.

THE DISEASES

There are two important leaf diseases of mint in Michigan, one caused by a *Septoria* and one caused by an *Alternaria*. The Septoria leaf spot has been studied by two Japanese workers (1) while the Alternaria leaf spot has not been investigated. The Alternaria and Septoria leaf spots have been identified in Michigan on the basis of the presence of *Alternaria* spores observed on collected specimens in the case of the former, and the presence of pycnidia containing typical *Septoria* spores in the latter. Also, isolations have been made by Dr. Ray Nelson from both types of leaf spots, one yielding an *Alternaria* and one a *Septoria*.

Review of Literature

Hemmi and Kurata (1), reported and described a leaf spot disease of mint in Japan caused by *Septoria*, and in their paper the species name *menthae* is ascribed to the fungus. Symptoms of the disease are described and are similar to those found on American mint. A different type of symptom is described, but also attributed to *Septoria*, but the description and illustration appear more like the Alternaria leaf spot. These workers report that 28° C. is optimum for germination of spores while 24° is optimum for growth of mycelium.

The Septoria leaf spot discussed in this thesis is probably the same species as that described by Hemmi and Kurata. Because this is not a certainty the fungus will be referred to as Septoria sp.

Economic Importance

The losses from mint leaf spot diseases are difficult to estimate. In some years there is practically no loss, while in others the plants may be also entirely defoliated. As the mint oil is distilled from the leaves, defoliation results in a direct loss of oil. In Oregon, experimental vacuum devices to sweep up the fallen mint leaves have been tested. In some mints there is a natural partial defoliation due to etiolation but major leaf losses may be caused by leaf spot.

Symptoms

Septoria Leaf Spot

The typical necrotic lesions caused by Septoria sp. begin in early August as small, pinpoint, dark brown spots on the leaves, enlarging to round or angular spots mostly from 1 - 5 mm. in diameter. Some remain pinpoint in size. The mature lesions are characteristically ashen-gray in color bounded by a narrow, dark brown perimeter. Around the margin of the lesion there is often a zone of faint chlorotic tissue extending a few millimeters into the healthy green tissue. In the greenhouse, color of the spots is often more brownish. The number of spots on a leaf may vary from one to many. In a particularly heavy infection, several may coalesce forming a large lesion. The affected leaves tend to remain attached to the stem, but defoliation may occur in case of heavy infection. The lower leaves are most severely attacked. The greater shade and resultant higher humidity nearer the soil provides optimum conditions for incubation and infection. Also, spores from pycnidia on the overwintered leaves on the soil are carried by splashing rain to the lowermost leaves of the young plants from whence the disease spreads upward as the plants grow. Pycnidia are soon produced on the upper surface of the leaf. During wet weather the needle-shaped conidia ooze from the ostioles of the pycnidia and these spores are spread by rain or irrigation to new infection courts.

Alternaria Leaf Spot

The Alternaria leaf spot is of a different type from that caused by *Septoria* sp. It first appears in June when the plants are young and begins with a small brown spot on a leaf which enlarges rapidly covering a large part of the leaf, usually more than half. The center of the lesion dries and turns brown while the edge remains yellow. Apparently, a toxin is produced by the fungus causing the yellowing of the leaf because of its rapid increase and the fact that the necrotic area is often very small compared with the chlorotic area. Before the whole leaf becomes yellow it usually falls off. This disease causes much more defoliation than the Septoria leaf spot. It spreads most rapidly in warm, dry weather probably because its spores are wind-borne. In inoculation experiments in the greenhouse the resultant infection was of a somewhat different nature. The early lesions were usually of a water-soaked purplish appearance rather than yellow, later turning brown. Alternaria leaf spot, like Septoria leaf spot, usually begins on the lower, older leaves and spreads progressively upward, often resulting in serious defoliation.

Etiology

Isolations of Leaf Spot Fungi

Leaves with Alternaria and Septoria leaf spots were removed from Scotch spearmint plants and washed for one-half hour under running tap water. The leaves were then cut into small pieces, each including a portion of the margin of a lesion. These pieces were surface sterilized for one minute in a 1:1000 mercuric chloride solution, following which they were rinsed in four changes of sterile distilled water and planted in Petri dishes containing prune agar. A few drops of 5% lactic acid were added to some of the plates before inoculation and this was effective in reducing bacteria growth. Transfers were made of the different fungi growing out of the tissues on the Petri plates to tubes of prune agar. Nine isolates were obtained. Several were morphologically similar, but all were retained in order to be sure that all fungi present were recovered. This method of isolation was not satisfactory. There was much mycelial growth of several fungi from the pieces of leaf tissue planted on the agar plates. A chlorazene disinfesting method used later proved a much better and simpler method. However, due the lateness of the season, no new material for isolation was available at this time. No Septoria was isolated in these attempts.

In order to obtain a pure line of each of the nine isolates, hyphal tip excisions were made. This was done by the following method. A small amount of hot prune agar taken directly from the steamer was poured into each of several Petri dishes which had been warmed over a Bunsen flame immediately before pouring. By doing this, a thin film of agar could be obtained through which hyphae would grow in a very thin layer. One could thus be more easily separated from the rest. Subcultures were made to Petri dishes of prune agar and after several days, hyphal tips were excised and transferred to slants of potato dextrose agar. The cutting instrument was an inoculating needle which had been flattened on a piece of iron with a hammer until thin and sharp. A Petri dish was opened, examined under a binocular microscope, and a hyphal tip protruding beyond the mass of mycelium was located and severed. It, together with a bit of adhering agar, was transferred to a slant of potato dextrose agar. This was done several times for each isolate.

Isolations were made by Mrs. Marie Mooar from Scotch spearmint leaves collected in the field, and yielded a fungus which was identified as a *Septoria*. Several isolates were contributed by her. Spore dilution plates were made and single colonies were selected and transferred to slants of potato dextrose agar. These were assumed to be pure lines, and were used in inoculation tests for pathogenicity.

Pathogenicity Tests

Test No. 1. Mycelium was used as inoculum in the first attempt to prove pathogenicity of a *Septoria* and the other isolates. Distilled water was poured into a culture tube of each isolate and the surface mycelium was removed by scraping gently with a sterile inoculating needle. The mycelial suspensions were poured into clean test tubes and triturated with a large stirring rod. Some of the suspensions contained spores in addition to the mycelium. Inoculations were made by brushing the suspensions on leaves of Scotch spearmint plants with sterile camel's hair brushes. Two plants were inoculated with each isolate, one on the top of the leaves and stem, and one on the under side of the leaves and stem. After inoculation, the plants were placed in a moist chamber, left 48 hours and then removed to the greenhouse bench.

No disease developed on the plants inoculated with *Septoria* sp. On the fifth day after inoculation, the plants inoculated with isolates No. 3 and No. 6 showed browning of portions of some of the leaves and also abscission of the most severely infected leaves. The reaction was similar to the *Alternaria* infections observed in the field, but did not extend farther in the leaf, as is typical of the *Alternaria* infection. No reactions were observed on plants inoculated with the other isolates.

Test No. 2. Another attempt was made to produce infection using six more isolates of *Septoria* sp. from Scotch spearmint. These isolates were growing in tube cultures and spores could be seen oozing from pycnidia in all tubes. A distilled water suspension of spores and mycelium prepared from each isolate was poured into separate beakers and more distilled water was added. A bit of Dreft was added to the spore suspensions in each of the six beakers to lower surface tension as mint leaves are difficult to wet. The leaves of three plants were dipped in each suspension and placed in lamp chimney moist chambers. Ground glass plates sealed the top and bottom and the inside was lined with moistened blotting paper. The three plants inoculated with each isolate were placed in separate moist chambers as were the untreated check plants. After 48 hours, they were removed from the moist chambers and placed on the greenhouse bench. No leaf spots developed on any of the plants.

Test No. 3. Although isolates No. 3 and No. 6 were apparently pathogenic it was decided to test the nine isolates again, this time using spores as the inoculum for those isolates producing spores and triturated mycelium for the isolates not producing spores. *Septoria* sp. was not included in these tests. Isolates Nos. 1, 3, and 4 produced no spores. Isolate No. 2 produced an abundance of unidentified conidia. Isolate No. 6 produced small, one celled, hyaline conidia in pycnidia. This fungus grossly resembled *Phyllosticta*. Isolates Nos. 5, 7, 8, and 9 were *Alternarias* producing abundant conidia. Distilled water spore suspensions were made from slant cultures of the fungi. These were filtered through two thicknesses of cheesecloth and examined under the microscope for spores which were present in all cases. The spores were applied to the spearmint plants by use of bulb atomizers. A little Dreft was added to each spore suspension and three plants were sprayed with each on both the upper and lower surfaces of the leaves. The aerial mycelium of isolates Nos. 3 and 6 was removed and ground as in the preceding experiment. Each suspension, and also a small amount of Dreft was poured into a beaker. Three Scotch spearmint plants were dipped into each mycelial suspension. The inoculated plants and three untreated plants were placed in lamp chimney moist chambers. After 48 hours the plants were removed from the moist chambers and placed on the greenhouse bench. Five days after inoculation, two of the plants inoculated with isolate 3 showed a few brown dead areas on the margins of the leaves. Only two or three leaves of each of the two plants showed this reaction. This reaction did not progress further nor did it change. The plants inoculated with isolate No. 6 and with the other isolates showed no reaction.

Though a reaction occurred on two plants inoculated with isolate No. 3, it was not a typical *Alternaria* type of infection because of its dark color and lack of progression when the plants were removed from the moist chamber. At this stage in the research the reaction was still considered inconclusive evidence of the pathogenicity of the organism due to its atypical nature. Because isolate No. 6 produced no reaction at all this time and the reaction caused by isolate 3 was very slight, it was thought desirable to repeat the inoculations once again without the Dreft, as its presence might have had some toxic effect on the spores and mycelium.

Test No. 4. The preceding experiment was repeated using spores as the inoculum for isolates Nos. 5 to 9, and triturated mycelium for the others. More concentrated mycelial suspensions were used in this experiment. Isolate No. 2 had not produced sufficient spores to warrant their use as inoculum in this case. *Septoria* isolates were included in this experiment in a third attempt to achieve infection with this fungus. Spores were used as the inoculum. To increase the concentration of the spore suspensions they were centrifuged and the supernatant liquid pipetted off. Inoculation was done with sterile camel's hair brushes, three plants being inoculated with each isolate. Untreated checks were included. The plants were placed in the moist chamber for 44 hours and then removed to the greenhouse bench. No plants inoculated with Septoria developed any disease. Three days after inoculation, the plants inoculated with isolate No. 3 again showed brownish spots on the leaves where pieces of mycelium were adhering. These did not change in size or color. Seven days after inoculation, one of the three plants inoculated with isolate No. 6 produced a blackened area at the margin of one leaf.

In spite of the failure to produce infection with Septoria it was believed that all the isolates must be pathogenic as few saprophytic Septorias are known and contamination by Septoria is rarely encountered. It was assumed that the technique of inoculation was not yet correct. Isolate No. 3 was considered to be pathogenic, though apparently not especially virulent and not producing a type of infection comparable to the Alternaria leaf spot in the field. Especially marked was the tendency of the infection to stop spreading in the leaf after the plants were removed from the moist chamber. The fungus itself had not produced a single conidium in culture and no spores were observed in scrapings from leaves infected by it, so identification was impossible. It was not known whether the fungus was sterile or whether the medium or environmental conditions were not correct for sporulation. Isolate No. 6 was believed to be an extremely weak pathogen but whether it was pathogenic at all was not certain.

Owing to the difficulty of obtaining infection with Septoria sp. and isolate No. 6, it was decided that some spore germination tests should be tried with several stimulants in addition to distilled water. One of the Alternarias, isolate No. 8, was also included. While realizing that a stimulant, if effective, would not duplicate natural conditions, it was thought that it should be tried in an attempt to establish pathogenicity. A stimulant could not conceivably endow pathogenicity to a spore which did not already have the capacity to infect, but it might increase the chance for the spores to infect by causing a more rapid and vigorous germination. The stimulants used were:

1. Nutrient broth (3 g. beef extract and 5 g. peptone per liter of water)
2. Potato extract (400 g. per liter of water)
3. Yeast extract (0.3%)
4. Sodium citrate (0.001%)
5. Distilled water (check)

A one-month old agar culture of Septoria and three-week old cultures of isolates Nos. 6 and 8 were used to provide the inoculum. Distilled water suspensions were prepared and filtered through two thicknesses of cheesecloth and the concentration of spores was adjusted. Glass slides were cleaned thoroughly with Bon Ami, and a thin layer of vaseline applied to one surface. Two small, round cover glasses were appressed to the vaselined side forming a rim about the cover glass. One drop each of the spore suspension and stimulant was added to each cover glass. Treatments were replicated twice. The control consisted of one drop of the spore suspension only. Two slides were placed in a Petri dish with a moistened filter paper in the bottom. The Petri dishes were placed in moist chamber dishes with a small amount of water in the bottom.

The slides were examined under low power of the microscope after 20 and 40 hours. Germination was noted on a comparative basis and is tabulated in Table 1.

Table 1

COMPARATIVE EFFECT OF STIMULANT SOLUTIONS ON GERMINATION OF SPORES OF THREE MINT LEAF SPOT ISOLATES

Stimulant Solution	No. 6		No. 8		*Septoria* sp.	
	20 hours	40 hours	20 hours	40 hours	20 hours	40 hours
Nutrient Broth	+++++	+++++	++++	+++++	++	+++
Potato Extract	+++	++++	++++	+++++	++	+++
Yeast Extract	+++	+++	+++	++++	++	+++
Sodium Citrate	+	+	++	+++	+	+
Distilled Water	-	-*	++	+++	-	-*

* Very slight germination.

All the materials stimulated germination of the spores more than distilled water. Nutrient broth, potato extract and yeast extract were nearly equal in their stimulatory effect on *Septoria* sp. spores. Nutrient broth proved the most stimulating medium for the germination of the spores of isolate No. 6. None had germinated in distilled water after 40 hours. Although the *Alternaria* spores germinated well in distilled water, germination was increased by the test solutions.

Test No. 5. Spearmint plants were inoculated with *Septoria* and the nine isolates using nutrient broth as the spore suspension medium. Triturated mycelium was used as the inoculum for isolates Nos. 1, 2, 3, and 4 and spores for Nos. 5, 8, and *Septoria* sp. A mixture of spores and mycelium were used for isolates Nos. 6, 7 and 9. Untreated checks were included. Three plants were inoculated with each isolate using camel's hair brushes. One was removed from the moist chamber after 48 hours, one after 60 hours, and one after 72 hours. After seven days all the plants which had been inoculated with *Septoria* sp. showed typical *Septoria* spots. The spots did not appear in large numbers but pathogenicity was established. After only 48 hours, the three plants inoculated with isolate No. 3 were heavily infected. Purple spots were present on the leaves and stem, and the stem of one of the plants was decaying. Thus it seemed that No. 3 was definitely infectious. After 72 hours small spots were observed on the plants inoculated with isolate No. 6. Once again infection, if any, was very slight and one could not be sure that the spots were fungous lesions. None of the other seven isolates produced a lesion of any kind and were apparently non-pathogenic.

Test No. 6. An experiment was conducted to confirm the pathogenicity of isolates Nos. 3, 6, and the *Septoria* used in the previous experiment, and to compare inoculations with broth and distilled water as suspension media. Spores were used as inoculum for *Septoria* and mycelial suspensions were prepared for isolates Nos. 3 and 6. One-month old cultures of *Septoria* and three-weeks old cultures of isolates Nos. 3 and 6 were used. Four plants were inoculated with each of the three suspensions by brushing the inoculum on the leaves. One plant was removed from the moist chamber after 12 hours, 24 hours, 48 hours, and 84 hours.

No infection occurred on the plants inoculated with *Septoria* sp. and removed after 12 and 24 hours. The plant removed from the moist chamber 48 hours after it was inoculated with the water suspension showed no spots, while the plant inoculated with the broth suspension produced many spots. Both plants removed after 84 hours were infected but the one inoculated with the broth suspensions was most severely infected.

It is not clear why the early attempts at infection with *Septoria* failed, for in this last experiment infection occurred with the use of a distilled water spore suspension on plants removed after 48 hours in the moist chamber. In the first three experiments plants were removed after 44 and 48 hours without having become infected. Low temperature in the greenhouse may have been a factor. The first experiments were done in January when the temperature was often very low in the greenhouse, especially at night, while the later ones were done in March when the warmer weather raised the temperature. Perhaps at the lower temperatures 48 hours was insufficient time to permit penetration of the germ tubes into the leaf tissue. The ground mycelium used in the first attempt may have differed from spores in its requirements for producing infection. Ground mycelium is not a natural inoculum, but should under ideal conditions be capable of infecting a susceptible host. In the second experiment a small amount of Dreft was added to the spore suspension to increase its wetting ability. Even such a small amount may have had a toxic effect on the spores. Dreft is known to inhibit germination of spores of certain organisms. Other factors such as density of spore suspension, age of spores, etc., would not likely be so sensitive as to adversely affect infection in all three attempts. There was no difference in the amount of infection on broth- and water-inoculated plants with isolates Nos. 3 and 6. Infection occurred with both isolates on plants removed from the moist chamber in 24 hours. The number and size of lesions produced by both fungi increased with prolonged time in the moist chamber. The plants inoculated with isolate No. 3 and left in the moist chamber 84 hours were defoliated. Those inoculated with isolate No. 6 always produced fewer, smaller spots than No. 3. In all cases infection did not progress to a noticeable extent after the plants were removed from the moist chamber.

Reisolations

In order to prove that an organism is an agent of infection it is necessary, in addition to its isolation and production of disease symptoms in the host, to reisolate the organism and identify it in pure culture as the original isolate.

Isolations were made from several leaves infected by the *Septoria* isolates. All the leaves were rinsed for one-half hour in running tap water. Four methods of isolation were used.

1. Leaves were soaked for five minutes in 1% chlorazene solution.
2. Leaves were soaked for ten minutes in 1% chlorazene solution.
3. Leaves were soaked for five minutes in 1% chlorazene solution and rinsed for five minutes in sterile distilled water.
4. Leaves were soaked for ten minutes in 1% chlorazene solution and rinsed for five minutes in sterile distilled water.

Following each treatment small pieces were aseptically cut from the margins of lesions and planted on prune agar in Petri dishes. After a few days there was enough growth of the fungus from the planted tissue to identify it as a *Septoria*. The fungus grew out of the tissue following every treatment. This constituted proof of the pathogenicity of the *Septoria* isolates.

Leaves infected with isolate No. 3 were removed from Scotch spearmint plants and reisolations were made for final proof of pathogenicity. The leaves were rinsed for fifteen minutes in running tap water, and divided into two groups. One was soaked for five minutes in 1% chlorazene solution, and one for ten minutes in 1% chlorazene solution. A small amount of detergent was added to the solutions to lower the surface tension. Bits of infected tissue were removed aseptically from the margins of the lesions and were transferred to Petri dishes of prune agar. The next day the fungus was growing from all pieces of tissue and appeared to be the same as the original isolate. Like the original isolate it produced no spores.

Growth Characters of Pathogens

The *Septoria* fungus grown on potato dextrose agar assumes the characteristic *Septoria* type of colony. It grows very slowly, becoming a tough, black, knotty mass of mycelium with ridges and depressions, nearly devoid of aerial mycelium. Here and there may be scattered white or gray patches of mycelium. Pycnidia form readily on the black stromata which are produced by a part of the vegetative mycelium. About four weeks after inoculation of tubes of potato dextrose agar, white masses of spores can be seen exuding from the ostioles of the pycnidia. These are typical *Septoria* spores.

Isolate No. 3 produces ample dark gray aerial mycelium typical of Alternaria on potato dextrose agar. The mycelium appressed to the bottom of the culture tube or Petri dish is black except for the lighter growing edge. It may sector producing white mutants. On Coons' agar the mycelium is cream-colored but may form dark gray sectors or "caps." The cream-colored mycelium does not produce aerial mycelium, as does the dark, and is slower growing. Spores were not produced on any medium by this fungus or by the single hyphal tip isolates from sectoring colonies.

Isolate No. 6 produces mycelium very similar to that produced by isolate No. 3. The color of the mycelium is dark gray but is somewhat less profuse than that produced by isolate No. 3. Pycnidia are produced which contain small, hyaline, oblong conidia. After three months in culture this fungus ceased producing spores.

Attempts to Induce Sporulation of Isolates Nos. 3 and 6

Isolate No. 3 created several problems because of its apparent sterility. First, it could not be identified as it had not produced either sexual or asexual spores, although it was isolated from lesions of the type usually associated with the Alternaria organism, and the character of the mycelium was of the Alternaria type. Secondly, it could not be used for inoculation experiments to determine the optimum temperature for infection or the minimum time for infection at 100% humidity. The mycelium is not a natural inoculum and for infection tests, it is unsatisfactory. As it was the only isolate of its type which was pathogenic, and as it was winter when further material for isolations was not available, attempts were made to induce it to sporulate. It was thought that there might be an optimum temperature for sporulation of the fungus, but when Petri plates containing Coons' agar were inoculated with the fungus and grown at ten different temperatures ranging from 10 to 30° C., no spores were produced.

Isolate No. 6 ceased producing pycnidia in culture. It, too, failed to produce spores when grown at temperatures ranging from 10 to 30° C. (Optimum temperatures for growth of isolates Nos. 3 and 6, and *Septoria* sp. are discussed in a later section.)

Isolates Nos. 3 and 6 were grown on several different media as it is well known that certain fungi will sporulate well on certain media and not on others. The media used were potato dextrose agar with 20 g. dextrose, 10 g. dextrose, and no dextrose; malt agar; prune agar; corn meal agar with 0.5% yeast extract; a medium containing sorbose, yeast extract, glycine, and malt extract. No spores were produced.

The host plant itself was used as a medium as it was thought that if the fungus would sporulate at all it should do so on its natural host. Two methods were tried.

1. Woody stems of Scotch spearmint were cut into lengths of about 3 inches and five or six of these were placed in each of several large test tubes containing a small amount of distilled water. These were plugged and autoclaved for fifteen minutes at fifteen pounds pressure. Bits of mycelium from tube cultures of isolates No. 3 and No. 6 were transferred to the pieces of spearmint stem.
2. Leaves of spearmint were removed from mature plants and surface disinfected for fifteen minutes in 1% chlorazene solution after having been rinsed for one-half hour in running tap water. They were then placed in 1 liter flasks which had been filled to a depth of about 1½ inches with muck and steamed for 45 minutes in the Arnold Steamer. After cooling, they were inoculated with bits of mycelium from isolates No. 3 and No. 6.

No spores were produced by either fungus though the mycelium grew well on the tissues and decayed them. Further work with isolate No. 6 was abandoned because of the weak pathogenicity of the organism.

A further attempt was made to induce sporulation of isolate No. 3 by using method 2 above. This time the newly reisolated No. 3 was used in place of the old stock culture. One flask was prepared as described and a second was prepared without steaming the leaves, but was inoculated directly after surface sterilization. The fungus grew in both cases but much more rapidly on the steamed leaves. No spores were produced.

Variants of Isolate No. 3

A peculiar phenomenon in the growth of the mycelium of No. 3 was observed on the Petri dishes used in the temperature experiment mentioned in connection with attempts to induce sporulation. On potato dextrose agar and prune agar, the aerial mycelium of this fungus is always a dark gray but the mycelium appressed to the bottom of the Petri dish or culture tube is black. Growth on the Petri plate from which the temperature plates were inoculated was entirely of this dark type. These plates contained Coons' agar. The fungus grew as a cream-colored mycelium at first, but soon vigorously growing, dark-gray sectors appeared in some of the plates. This dark mycelium grew faster than the white and at all but the highest temperatures formed "caps" at one or two places on the periphery of the white growth. At 30 and 32° C. the dark variant was in the form of a regular triangular sector.

This indicated that isolate No. 3 was not a pure line. There were at least two morphological types of mycelium present, the black and the white. Bits of the mycelium from a Petri dish culture of isolate No. 3 were transferred to Petri dishes of potato dextrose agar. This plate, it is remembered, appeared black. When the plates were covered by the mycelium, most of each colony was black, but white sectors and various intermediate sectors between black and white were present. It seemed likely, therefore, that some of the tubes of isolate No. 3 used in inoculations were also not pure and perhaps this may have had some effect on pathogenicity. This may explain the erratic results of the pathogenicity tests.

Single hyphal tip excisions were made from the sectored plates to test these isolates for pathogenicity. Transfers were made from various places on the sectoring plates to Petri dishes containing a thin layer of prune agar. Hyphal tips were then excised and these were placed in tubes of potato dextrose agar. Nine isolates were obtained, none of which produced spores. Up to this point it was thought that the dark mycelium was the pathogenic strain while the white was non-pathogenic. Tests had been made of each and the black mycelium caused infection while the white did not. The aerial mycelium of each of the nine isolates was triturated separately in water in a mortar and brushed on the leaves of Scotch spearmint plants. Two plants were inoculated with each line. The plants were placed in the moist chamber for 36 hours after which time those inoculated with Nos. 4, 6, 7, and 8 showed the typical spots on the leaves. Lines No. 6 and No. 8 produced a more severe infection than No. 4 and No. 7. None of the other lines was pathogenic. On examining the stock cultures of these isolates and checking their morphological characters with their pathogenicity, it became clear that there was no correlation between color of mycelium and pathogenicity. Some of the pathogenic lines were dark and some light, and some non-pathogenic lines appeared exactly like pathogenic ones.

New *Alternaria* Isolations

Since all attempts at inducing spore production in isolate No. 3 had failed, an attempt was made to isolate the fungus again from plants infected in the greenhouse and in the field. The leaves of a clone of *Mentha spicata* collected in the state of Washington and growing in the greenhouse were badly infected by a leaf spot, apparently of the *Alternaria* type. After detaching newly infected leaves and washing them for twenty minutes in running tap water, they were surface disinfected by soaking for three minutes and five minutes in 1% chlorazene followed by a rinsing in sterile distilled water for five minutes. Pieces of infected tissue were aseptically cut from the lesions and planted on prune agar. After 48 hours mycelium was growing from some of the pieces and transfers were made to potato dextrose agar slants. Leaves soaked for five, eight, and ten minutes without subsequent rinsing yielded no fungus.

The fungus isolates were sporulating *Alternarias* and were not accompanied by any contaminants. Four hyphal tip excisions were made. The spores and mycelium of these four pure lines were triturated in a mortar in a distilled water suspension and brushed on leaves of Scotch spearmint. The plants were placed in the moist chamber for 48 hours, after which time they were placed on the greenhouse bench. No infection was produced by these isolates. This was somewhat a surprise as the organism isolated was the same from each piece of tissue and no contamination was evident.

A row of hybrid peppermint plants in the field was being seriously defoliated by a leaf disease characteristic of Alternaria. Isolations were made from these leaves. The leaves were rinsed for one-half hour in running tap water as before. Those treated with 1% chlorazene and rinsed with sterile distilled water yielded the fungus when pieces were planted on prune agar as did those soaked for five minutes and planted on agar without rinsing. Leaves soaked ten minutes in chlorazene and not rinsed failed to yield the fungus. The fungus was identified as an Alternaria and appeared to be the same as the one isolated from the Mentha spicata clone. Transfers were made of the mycelium growing from several pieces of the tissue and six hyphal tip excisions were made by the method described before. All except one of these lines produced Alternaria spores. Three more lines were procured by single spore isolations. A spore suspension from three isolates was sown on each of three plates of potato dextrose agar and after germination several spores were picked out in each plate by observations with a binocular disecting microscope. Isolations were made by use of a fine glass capillary tube. The injected spore was driven from the tube into a slant of potato dextrose agar by heating the distal end of the capillary tube. These nine pure lines were tested for pathogenicity together with a repeat testing of the four lines isolated from the _Mentha_ _spicata_ clone. Original isolate No. 3 was also included. All cultures except one, which was apparently a contaminant, produced _Alternaria_ spores. A suspension of mycelia and spores was prepared and brushed on young spearmint leaves. Two plants were inoculated with each line and placed in the moist chamber for 72 hours together with untreated checks. Ice was added to the moist chamber twice a day to keep the temperature below 25° C. After removal from the moist chamber the plants were placed in dense shade out of doors to avoid the high temperatures of the greenhouse. No plants became infected except those inoculated with original isolate No. 3 which completely destroyed one plant and defoliated another. There is no apparent cause for non-infectiousness of these isolates. All except one were spore-producing _Alternarias_ and very similar organisms. No contamination was present in any of the isolation plates except for the one mentioned. A standard method of isolation was used, and yielded good results in many cases. The conditions for infection seemed ideal; 72 hours in the moist chamber is much longer than is needed to cause infection by original isolate No. 3. The writer believes that the possibility of pathogenic races in this leaf spot organism should be investigated. The inoculations were all made on Scotch spearmint from which the only pathogenic isolate, No. 3, was recovered. The new isolates were made from an unidentified clone of *Mentha spicata* and a hybrid peppermint. Perhaps these strains are biological races that differ from the one on Scotch spearmint and will not cross infect.

Environmental Relations

Effect of Temperature on Growth of Pathogens

An experiment was conducted to determine the optimum temperature for growth of the mycelium of the three pathogens. A Petri dish of potato dextrose agar was seeded with a suspension of *Septoria* spores, and others were inoculated with mycelial transfers of isolations Nos. 3 and 6. The mycelium was allowed to grow until the entire surface of each plate was covered. Filter paper discs 7 mm. in diameter were sterilized in a 10% glucose solution in the autoclave. The discs were transferred aseptically to the Petri dishes and the mycelium was allowed to grow through the paper discs for several days. These discs constituted a uniform inoculum for the temperature studies. Sterile Petri dishes were each poured with 20 cc of Coons' synthetic agar. One paper disc was transferred aseptically to the center of four plates. The plates were placed in incubators at the following temperatures: 10, 14, 18, 22, 24, 26, 28, 30, 32, and 34° C. After 18 days the mean diameter of each Septoria colony was measured, and the average diameter of the four colonies was calculated for each temperature. This is shown in Table 2.

Table 2

DIAMETERS OF COLONIES OF SEPTORIA SP. GROWN AT DIFFERENT TEMPERATURES

Degrees C.	Millimeters				
	Plate 1	Plate 2	Plate 3	Plate 4	Average
10	2	2	3	2	2.3
14	7	7	7	7	7.0
18	9	8	9	*	8.6
22	15	10	11	13	12.3
24	13	12	12	11	12.0
26	11	15	12	11	12.3
28	10	11	10	25**	10.3
30	9	8	7	6	7.5
32	3	4	3	4	3.5
34	0	0	0	0	0.0

* Contaminated.
**Apparently a mutant, not included in average.

From the table it can be seen that the optimum temperature range for growth of the mycelium is from 22 to 26° C. with a gradual decrease in the rate of growth at higher and lower temperatures. The highest temperature at which growth occurred was 32° C. Very slight growth occurred at 10° C. The diameters of colonies of isolate No. 6 were calculated after 10 days and the results are as tabulated in Table 3.

Table 3

DIAMETERS OF COLONIES OF ISOLATE NO. 6 GROWN AT DIFFERENT TEMPERATURES

Degrees C.	Millimeters				
	Plate 1	Plate 2	Plate 3	Plate 4	Average
10	29	28	28	28	28.3
14	48	47	48	49	48.0
18	67	66	68	68	67.3
22	68	68	70	69	68.8
24	61	60	64	63	62.0
26	53	55	59	57	56.0
28	23	28	41	48	35.0
30	13	16	18	17	16.0
32	5	3	1	0	2.3
34	0	0	0	0	0.0

The optimum temperature for isolate No. 6 is apparently between 18 and 22° C., though growth was excellent from 18 to 26° C. At temperatures above and below this range, the rate of growth decreases rapidly. No growth occurred at 34° C.

The diameters of the colonies of isolate No. 3 grown at the different temperatures are recorded in Table 4.

Table 4

DIAMETERS OF COLONIES OF ISOLATE NO. 3 GROWN AT DIFFERENT TEMPERATURES

Degrees C.	Millimeters				
	Plate 1	Plate 2	Plate 3	Plate 4	Average
10	32	29	32	29	30.5
14	49	47	46	**	47.3
18	68	67*	69*	**	68.0
22	70	69	73	**	70.7
24	69	67	61*	**	65.7
26	51	**	**	**	51.0
28	49	47*	**	**	48.0
30	18	21*	25*	26*	22.5
32	4	6*	7*	8*	6.3
34	0	0	0	0	0.0

* Sectoring colony, but measurable.
** Sectoring colony, but not measurable due to extent of sector.

The optimum temperature for the growth of the mycelium of isolate No. 3 also appears to be between 18 and 22° C. Rapid growth also occurred at 24° C. This gives evidence of a definite optimum range from 18 to 24° C. The growth rate tapers off at temperatures above 24 and below 18° C. No growth occurred at 34° C. Many of the colonies produced the variants which were discussed in another section.

The variants appeared in the forms of "caps" on colonies grown between 14 and 30° C., and as sectors on those grown at 32 and 34° C. The "caps" interfered with measurements of the diameters of the colonies. Where the diameter was obscured, a measurement was not taken but where the "cap" had not yet covered an arc of 180° the measurement was taken.

Effect of Temperature on Germination of Spores of *Septoria* sp.

An experiment was done to determine the optimum temperature for germination of *Septoria* spores. Several slides were cleaned with Bon Ami after which they were covered with a clear, thin layer of 2% water agar. After the agar had solidified, a few drops of a distilled water suspension of *Septoria* spores were placed on each slide and allowed to spread evenly. The slides were placed in Petri dishes lined with moist filter paper in the top and bottom. One Petri dish with a slide was placed in each of 10 incubators set at temperatures of 10, 14, 18, 22, 24, 26, 28, 30, 32, and 34° C. After 36 hours the slides were removed and examined under the microscope. The percent germination for each temperature was determined by examination of 100 spores. Very few lateral germ tubes were produced on spores germinating at temperatures above 18° C. Germination was estimated by determining whether there was an increase in length of the spore (terminal germ tube) compared with ungerminated spores. At temperatures of 18° C. and below, note was made both of lateral germ tubes which were frequent, and of terminal increase in length.

Germ tube lengths were also calculated for spores germinated at all temperatures. This was done by measuring the length of spore plus its terminal germ tube. This had to be done as it was impossible to distinguish the limits of the spore and where the terminal germ tube began. 20 to 30 spores were measured on each slide. These measurements were averaged for each temperature. A mount of fresh Septoria spores was made on a slide coated with water agar and the average length of 20 of these was taken to be standard for ungerminated spores. This figure was subtracted from the average measurements of the spores and germ tubes for the different temperatures. Results appear in Table 5.

Table 5

EFFECT OF TEMPERATURE ON GERMINATION OF SPORES OF SEPTORIA SP.

Degrees C.	% Germination	Average Length of Germ Tube
10	0	2.7
14	12	6.4
18	87	70.6
22	91	134.6
24	89	125.2
26	90	88.9
28	90	104.5
30	76	77.2
32	34	25.9
34	0	- 4.5

The percent germination was highest from 18 to 28° C., there being no appreciable difference in any of the figures in that range. Below 18°, there was a sharp drop in germination. No germination occurred at 10° C. The drop above 28° C. was more gradual and at 34° C. no germination was noted.

The optimum temperature for growth of germ tubes was from 22 to 24° C. Germ tubes also showed good enlargement at 26 and 28° C., but were somewhat shorter than those germinated at 22 and 24° C. There is a sharp drop in average length of germ tubes below 22 and above 28° C. At 34° C. the average lengths of spores measured were shorter than the average of the freshly harvested spores.

Time-Temperature Relations for Infection by *Septoria* sp. at 100% Humidity

On the basis of previous experiments, germination of *Septoria* spores is best at 22 to 28° C. and growth of mycelium is best at 22 to 26° C. In infection tests to determine the optimum temperature for infection, incubators set at temperatures of 18, 22, 24, 26, 28, and 30° C. were used.

Moist chambers were prepared and placed in each of the six incubators. These consisted of glass cylinders closed at one end and lined with moistened paper toweling. These were inverted over pans of water so as to make a seal and provide moisture for the chambers. These were placed in the incubators and left for eight hours before the inoculated plants were placed in them to insure their having reached a constant temperature.

A concentrated spore suspension was prepared from several cultures of one-month old *Septoria* sp. by flooding the slant with water and shaking the tubes to release the spores. The suspension was filtered through two thicknesses of cheesecloth to remove bits of mycelium and was then applied to the upper and lower surfaces of the leaves of Scotch spearmint plants with an atomizer after first wetting them with distilled water. Three plants were placed in each moist chamber.

The plants were removed after 36 hours. In previous experiments, infection had never occurred using distilled water, when plants were in the moist chamber for 48 hours or less. However, it was thought that 36 hours would be adequate for extension of germ tubes long enough to penetrate the mint leaves at the optimum temperature for infection. On the basis of spore germination tests, the germ tubes at the optimum temperature range could be expected to extend over 100 microns in 36 hours and many measured well over 200 microns. It was thought that if plants were left in the moist chamber for too long a time, they would become infected too heavily and determination of the optimum temperature would be impossible. In nature, the disease often spreads very rapidly through a field. For such a rapid spread to occur a short incubation time must be possessed by *Septoria*.

After removal from moist chambers the plants were placed on the greenhouse bench and left for observation. No spots developed on any of the leaves. It was concluded, therefore, that 36 hours incubation time, even at the optimum temperature range, was too short to permit infection.

The previous experiment was repeated, removing the plants from the moist chambers after 48 hours instead of 36 hours. Eleven days after the plants had been inoculated small dark spots were observed on all plants. After a few more days these enlarged to the typical sized *Septoria* spots. The greatest number of spots appeared on those plants incubated at 22, 24, and 26° C. though infection was fairly uniform over the whole range.

It was concluded from this experiment that the optimum temperature for infection of Scotch spearmint by *Septoria* sp. is from 22 to 26° C., though infection occurred above and below this range.

Further experiments were conducted to confirm the optimum temperature findings and also to determine the minimum time for infection of Scotch spearmint at 100% humidity. On the basis of the preceding experiment, this minimum time must be less than 48 hours. Plants were inoculated as before. Two moist chambers were prepared for each of four incubators at temperatures of 18, 22, 26, and 30° C. Four plants were removed from each incubator at the end of 36, 39, 42, and 45 hours.

After their removal from the moist chambers the plants were placed on the greenhouse bench. After 10 days a few spots were observed on the leaves of some plants, but it required 20 days for full infection to develop. At this time counts were made of the spots on the leaves and tabulated in Table 6.

Table 6

EFFECT OF DIFFERENTIAL TEMPERATURES ON INFECTION OF SCOTCH SPEARMINT PLANTS BY SEPTORIA SP. AT VARIOUS TIME INTERVALS. PLANTS MAINTAINED AT 100% HUMIDITY.

Exposure Period	Temperatures			
	18° C.	22° C.	26° C.	30° C.
	Number of Spots			
36 hours				
Plant 1	6	29	54	23
Plant 2	27	25	32	6
Total	33	54	86	29
39 hours				
Plant 1	60	59	3	30
Plant 2	41	34	18	5
Total	101	93	21	35
42 hours				
Plant 1	5	34	38	11
Plant 2	10	33	12	12
Total	15	67	50	23
45 hours				
Plant 1	20	24	0	22
Plant 2	40	5	6	9
Total	60	29	6	31
TOTAL	209	243	163	118

Infection occurred on all plants which were in the moist chamber for 36 hours. Infection had never occurred before on plants removed after this length of time and no explanation is apparent. The technique for this experiment and the preceding two were the same. Age of spores, concentration of spore suspension and condition of plants all were the same. The only apparent difference in the conditions of the last three experiments was the temperature of the greenhouse in which the plants were kept following their removal from the moist chambers. The experiment in which all the plants were removed from 100% humidity after 36 hours was done in April when the temperature in the greenhouse was often cool. The experiment in which the plants removed after 48 hours, and the one under discussion were done in June and July respectively when the temperature was often high on sunny days. Perhaps at too low a temperature, infection cannot take place even though the requirements of time and temperature at 100% humidity had been met. The greatest number of spots occurred on plants incubated at 22° C. Those incubated at 18° C. developed the next greatest number. The number of spots on those at 26° C. was still smaller, while the number developing on plants incubated at 30° C. was much smaller than at any of the other temperatures. These results, in general, confirm previous observations that the most severe infection by <u>Septoria</u> sp. usually occurs on plants in moist chambers at 22 to 26° C. In this experiment, however, more spots developed at 18 than at 26° C.

The experiment was repeated to confirm the results on optimum temperature for infection, and to determine whether plants removed after 36 hours in 100% humidity would again become infected. The same temperatures as before were used but the times of removal were changed slightly. Plants were removed after 36 hours, 40 hours, 44 hours, and 48 hours.

After removal from the moist chambers, the plants were placed in a shady place outdoors instead of in the greenhouse as it was thought that the extreme high temperature in the greenhouse, sometimes over 100° F., might have a deleterious effect on the development of the disease. Such a high temperature is not encountered in the field. A count of the lesions taken 12 days after inoculation is recorded in Table 7.

Table 7

EFFECT OF DIFFERENTIAL TEMPERATURES ON INFECTION OF SCOTCH SPEARMINT PLANTS BY *SEPTORIA* SP. AT VARIOUS TIME INTERVALS. PLANTS MAINTAINED AT 100% HUMIDITY.

Exposure Period		Temperatures			
		18° C.	22° C.	26° C.	30° C.
		Number of Spots			
36 hours -	Plant 1	0	7	27	4
	Plant 2	0	12	9	6
	Total	0	19	36	10
40 hours -	Plant 1	22	30	55	14
	Plant 2	22	33	6	11
	Total	44	63	61	25
44 hours -	Plant 1	3	19	36	0
	Plant 2	2	11	3	6
	Total	5	30	39	6
48 hours -	Plant 1	9	14	10	6
	Plant 2	1	22	0	13
	Total	10	36	10	19
TOTAL		59	148	146	60

The data show definitely that infection was most severe on plants in moist chambers at temperatures of 22 to 26° C. This, in general, confirms the results of the previous experiments, except that in this experiment infection on plants incubated at 18° C. is much less severe at 22 and 26° C. than in the previous experiment.

Infection again took place readily on plants removed from the moist chambers after 36 hours, showing that the minimum time for infection at 100% humidity is less than 36 hours.

SUMMARY

There are two common leaf spot diseases of Scotch spearmint in Michigan. On the basis of previous isolations and presence of spores on the lesions, one is apparently caused by a Septoria and the other by an Alternaria. Isolations made from the two types of spots yielded three pathogens: a Septoria; isolate No. 3, a non-sporulating fungus resembling Alternaria; and isolate No. 6, an unidentified, weakly pathogenic fungus producing spores resembling Phyllosticta.

Later isolations were made from a clone on Mentha spicata and a hybrid peppermint. These consistently yielded sporulating Alternarias which were not pathogenic on Scotch spearmint. It was conjectured that biological races of this fungus may exist.

The optimum temperature for growth of each pathogen was determined. They are: for Septoria, 22 to 26° C.; for isolate No. 3, 18 to 24° C.; for isolate No. 6, 18 to 22° C.

The optimum temperature for germination of Septoria spores is 22 to 24° C.

The optimum temperature for infection of Scotch spearmint by Septoria sp. is 22 to 26° C.

The minimum time for infection of Scotch spearmint by Septoria sp. at 100% humidity is less than 36 hours.

BIBLIOGRAPHY

1. Hemmi, Takewo and Shizuko Kurata. Studies on Septorioses of Plants. V. *Septoria menthae* (Thum.) Oud. causing the serious *leaf spot disease* of cultivated mints in Japan. 19 pp. 6 text figures. Contributions from the Laboratories of Phytopathology and Mycology, Kyoto Imperial University, Kyoto, Japan. No. 69. 1933.

2. Nelson, Ray. Verticillium wilt of Peppermint (*Mentha piperita* L.) Mich. Agr. Exp. Sta. Tech. Bull. 221, June 1950.