STUDIES ON THE SIGNIFICANCE OF THE MICROFLORA OF WILT AFFECTED CLOVER PLANTS

by

W. E. Sackston.

A THESIS

submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfilment of the requirements for the degree of Master of Science.

September - 1940.

STUDIES ON THE SIGNIFICANCE OF THE MICROFLORA OF WILT AFFECTED CLOVER PLANTS.

W. E. Sackston.

Abstract.

Field, laboratory, and greenhouse investigations were conducted on wilt and root rot of red clover.

Repeated isolations from tissues in various stages of disease yielded 3 important fungi:- *Verticillium albo-atrum*, *Fusarium avenaceum*, and *Sclerotinia trifoliorum*.

Temperature and pH relationships of these fungi in pure culture are reported.

Substances produced by the fungi in a liquid medium were shown to be toxic to red clover.

Inoculation experiments with the 3 fungi were performed. Each was found pathogenic under certain conditions.

TABLE OF CONTENTS.

STUDIES ON THE SIGNIFICANCE OF THE MICROFLORA OF WILT AFFECTED CLOVER PLANTS.

INTRODUCTION

Official figures for the years 1937 and 1938 give the total area sown to field crops in Quebec at over 6,000,000 acres, and place the value of these crops at about $81,000,000. Of this, 3,600,000 acres were sown to hay and clover, worth about $37,000,000. Hay and clover are the most important field crops grown in this province; it is obvious, therefore, that any studies which may lead to the production and use of varieties of these crops which are more suitable or give higher yields than those now in use, are of great importance to Quebec agriculture.

The Agronomy department at Macdonald College has been conducting breeding projects with the object of improving certain field and forage crops. In one of these projects an attempt is being made to select strains of red clover that will combine winter hardiness and disease resistance with other desirable characters. Fields of red clover have been known to remain productive several times longer than the usual 2 to 3 year period. The development of such a long-lived strain which could be grown throughout the clover areas in the province would be a great aid to farmers. Other workers have indicated that the short life of clovers may often be attributed to diseases, especially those caused by root-invading fungi. Winter injury and the effects of root pathogens of clover are very closely correlated. This thesis is an account of

studies conducted on Verticillium albo-atrum Reinke and Berthold, Fusarium avenaceum (Fr.) Sacc., and Sclerotinia trifoliorum Erikss., three fungi found associated with wilt affected clover plants at Macdonald College.

Wilt and root rot are two distinct diseases, caused by organisms which attack the plant in different ways. Wilt diseases are those in which the pathogen attacks the vascular system of the host and causes the top to wilt, without first destroying the crown or roots. Root rotting organisms attack the root and crown, and by causing their death induce a wilting of the tops. Although the diseases are etiologically distinct, first symptoms of both are shown by the foliage of the plants. Thus no careful distinction can be made between these two troubles of clover in this account. Where the foliage was seen to be wilted, the disease is referred to as 'wilt', regardless of cause; where dead or diseased plants were examined and the roots found decayed, the term "root rot" is used.

PRELIMINARY OBSERVATIONS

The studies herein reported were carried on from the spring of 1939 to the fall of 1940. They were based to a considerable extent, however, on preliminary observations made by the author and others during the summer and fall of 1938.

Work done by others in the summer of 1938 showed that in one spaced planting of red clover, set out by the Agronomy department, only 52% of the plants survived their first winter. Close examination of the remaining plants showed root rot to be one of the diseases present; the occurrence of sclerotia on these plants indicated that 2.3% of the plantation was affected by a species of Sclerotinia. Examination of the same plants in the fall failed to disclose any sclerotia, which had been plentiful earlier in the season.

Roots of most of the dead plants showed a white fungus fruiting on them. Microscopic examination and culture studies revealed the fungus to be Verticillium sp. It was used in subsequent inoculation and culture studies. Many other fungi were isolated in the course of repeated attempts to find the cause of the root diseases of clovers, but as none of them occurred consistently they were not studied.

FIELD STUDIES

Wilt Symptoms on Red Clover.

Clover plants suffering from either wilt or root rot show characteristic foliage symptoms. Young leaves generally show the trouble first; they become flaccid, then gradually dry out, becoming shrivelled. Their color changes slowly from green to yellow green, then as the leaves die it becomes brown or black. The petioles of affected leaves bend downwards, and the leaves droop. The petioles as well as the leaves may be streaked with brown or black. In stemmy plants, the lower leaves are affected before the terminal ones. Plants in an advanced stage of wilting appear scorched.

Variation in the symptom picture may be observed in individual plants; this is demonstrated in the photographs, Fig. 1 to Fig. 4. Another symptom observed in plants suffering from root troubles is stunting. The plant illustrated in Fig. 5 showed considerable root injury; the dwarfing effect of the disease may be seen by comparing it to the healthy plant in Fig. 6, photographed to the same scale.

Roots of plants which appear wilted vary greatly in appearance; the symptoms observed on the roots could not be correlated with the above-ground symptom picture. In some diseased plants, the root system looked healthy, and no lesions or vascular discoloration were observed; in some others, the tap root and laterals seemed sound, but the crown portion was discolored, or even dead and rotten. The roots of those plants which had died down completely were in many cases discolored and rotten. Stunted roots which appeared healthy in other respects were also observed.

Fig. 1. Crown of plant dead, showing drooping of leaves.

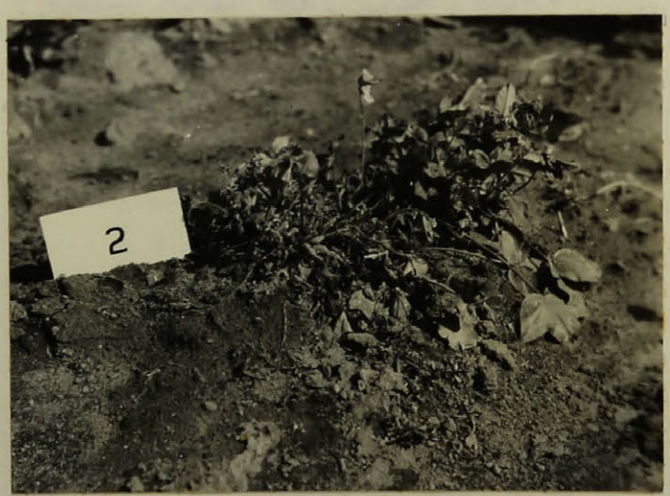

Fig. 2. Inner leaves dead, outer leaves green.

Fig. 3. Outer leaves dead and discolored, inner circle of leaves still green.

Fig. 4. Inner leaves dead, outer ones showing wilt at mid-day.

Fig. 5. Clover plant suffering from root rot.

Fig. 6. Healthy plant of red clover.

Review of Literature.

Wilting of red clover plants appeared to be of importance at Macdonald College, so a search was made through the literature to see what similar troubles of clover had been reported.

Chester (1890) described a clover disease caused by Sclerotinia trifoliorum; the disease was called variously stem rot, root rot, and wilt. Clover plants wilted completely to form dead spots in the field. Decay of the stems started at the soil level, causing the tops to wilt while the roots remained sound. Mycelium of the fungus occurred in dead tissues, and sclerotia were formed in and around the dead plants. Wolf and Cromwell (1919) reported that the stems and leaves of affected plants turned yellow, then quickly succumbed.

Cormack (1937) found that several species of Fusarium, including F. avenaceum, caused a typical root rot on alfalfa, sweet clover, and Trifolium spp.; the disease was particularly severe after winter dormancy of the hosts. Affected roots developed dark lesions, sometimes involving rotting of the entire root system, and weakening and death of the top.

Wilting of a large number of hosts is known to be caused by species of Verticillium; the only reference seen to Verticillium sp. on clover, however, was made by Boning (1936), who reported clovers as one of a series of crops to be avoided when using crop rotation as a control against V. dahliae on horse-radish. Verticillium wilt on herbaceous hosts is described by Rudolph (1931). The foliage may wilt during the day and recover at night to some extent, but gradual decline of the

plant continues until it dies. The leaves may hang limp, and tender shoot tips droop. The lower leaves may wither before the terminal ones are affected. The internodes may be shortened, causing stunting. In humid regions the fungus has been reported by Bewley (1922) to fruit at the base of dead plants. Roots of most host plants show no external lesions, but large, black, irregular cankers may appear on tender tap-rooted plants. Cross-sections of stems and roots of diseased plants usually show discoloration of the vascular tissues.

A close relationship has been found to exist between winter killing of clover plants and the activity of root pathogens. In the Canadian Plant Disease Survey report for 1936, a note from Alberta states that Sclerotinia trifoliorum is "not accountable for all winter killing noticed but clearly associated with many cases." Fergus and Valleau (1926) in Kentucky, concluded that much of the winter mortality of red clover must be closely connected with injury, its death being caused directly or indirectly by the factors producing the injury, such as fungi. Young (1923) found that winter loss of red clover may be increased by disease, and that "winter injury seems to be one of the conditions which predispose clover to attacks of the wilt." Dickson (1939), in his outline of forage crop diseases, states "the root rots of alfalfa, sweet clover and the clovers reported as caused by species of Fusarium are probably all closely associated with host plants injured by winter conditions or plants in a state of winter dormancy."

Disease Surveys.

Many farm fields of red clover were examined in the course of survey trips through the western part of Quebec. No wilt or root rot of clover was observed. Intensive surveys were conducted throughout the growing season on all the spaced plantings of red clover at Macdonald College. The clover plots at the Central Experimental Farm, Ottawa, also were visited in July, 1939.

A preliminary survey on 1 block of clover at the college in the summer of 1938 revealed sclerotia to be associated with most the clover plants showing root rot. Subsequent investigation by the author on the same and other blocks in 1938 and 1939 failed to disclose any sclerotia. None were seen in the plots at Ottawa. In the spring of 1940, however, sclerotia were found on some of the wilted plants examined in a spaced planting at Macdonald College.

Occurrence of Verticillium sp.

Surveys revealed Verticillium sp. fruiting on many diseased plants throughout the growing season. The fungus occurred on the basal portions of dead stems, on dead tissues of the crown, and on dead roots. It was never observed on healthy tissues, but was frequently found on the dead portions of plants which were still alive, especially when the living top showed wilt symptoms. It seems to grow only under moist conditions, for it does not appear higher than 1 or 2 inches up the stem, and is usually found on covered plant material. It has been seen only on red clover, usually near the soil level. The fruiting structure is a granular mass of spores and mycelium closely appressed to the host tissue; it is usually white, but pinkish colored spore

masses have been seen. The fruiting mycelium may occur in so small a mass that it can barely be seen with the naked eye; it rarely extends for more than a half inch.

Seasonal Development of Wilting.

Surveys made on a spaced block of 1,188 1-year old red clover plants in the middle of June, and again in the middle of August, in 1939, show that the disease situation in that block changed during the season, as indicated in the following table (table 1.). These plants had all survived the winter successfully. Plants labelled "healthy" were all those not showing wilting.

Table 1. Seasonal Development of wilt in red clover.

Description of plants.	As at June 15		As at August 10.	
	No.	Percent	No.	Percent
Missing	477	40.15	576	48.48
Dead	4	0.34	127	10.69
Wilt or Root Rot.	0	0.	38	3.20
Healthy	707	59.51	447	37.45

Winter killing

Detailed records of all the diseases present on a spaced block of 1404 red clover plants were made late in October, 1938, and compared with similar records made on the same block in the middle of May, 1939. The plants had been set out in the spring of 1938. The surveys were an attempt to correlate disease resistance of the plants with their ability to withstand winter injury. The data are presented in Table 2.

Table 2. Winter killing of red clover plants.

Description of Plants.	As at October 22, 1938.		As at May 16, 1939.	
	No.	Percent	No.	Percent
Missing	35	2.5	37	2.6
Dead	30	2.1	928	66.1
Diseased	173	12.2	291	20.7
Healthy	1166	83.0	148	10.5

Records for individual plants showed that no correlation could be worked out between diseases present in the fall and the winter hardiness of the plant. Plants which had seemed healthy in the fall were dead in the spring, and some plants which had appeared diseased survived. The same situation prevailed in plots examined in the fall of 1939 and spring of 1940. Winter killing was too severe and general to be correlated with the condition of the plants in the fall.

LABORATORY STUDIES

Isolation Studies.

Isolations were made from stem and root tissues of clover plants in various stages of wilting, and from dead plants, throughout the growing season. Tissue cultures were made from surface sterilized material and planted on potato dextrose agar in petri plates. In some tests the agar was acidified with several drops of 25% lactic acid. Suspensions of macerated surface sterile tissues were also plated. Results of the isolations from 35 plants, made at 3 different times during the season, were typical. Isolations from the superficial fruiting structures of Verticillium albo-atrum on dead tissues yielded pure cultures of this organism repeatedly. Where isolations were made from plants on which V. albo-atrum had been observed fruiting, it was the only organism which appeared consistently on the plates. It is rarely obtained from internal tissue transplants, but grows out from flamed tissues readily. Various species of Fusarium were isolated at different times, but no one species was found consistently in 1939. Numerous other fungi and bacteria developed on the plate cultures, but none of them appeared with sufficient regularity to justify further study. The conclusions drawn from isolations made in 1939 were that planting surface sterile tissue on agar was preferable to macerating the tissue; that slight acidification was sometimes helpful in reducing bacterial contamination; and that V. albo-atrum, although it appeared from only one third of the diseased or dead plants used for isolations, and was cultured only from those tissues on which it could be seen fruiting, was the only organism isolated with sufficient regularity to justify further studies.

Sclerotia were found on wilted clover plants by the author for the first time in the spring of 1940. Sclerotia were surface sterilized in mercuric chloride (1:1000) for from 10 to 20 minutes before being rinsed and inoculated onto agar slants. Three out of 4 sclerotia used in the first isolation, with 10 minutes surface sterilization, gave pure cultures of a Fusarium sp. later identified as F. avenaceum; the fourth failed to germinate. These results were duplicated independently by another worker in the same laboratory. Later isolations from other sclerotia gave pure cultures of Sclerotinia trifoliorum; F. avenaceum could not be isolated from sclerotia in other attempts.

Pure Culture Studies.

The 3 organisms which were studied were sent to specialists for identification. The Verticillium sp. was identified by G. H. Berkeley, of the Dominion laboratory of plant pathology at St. Catharines, Ont. On the basis of cultural characteristics he determined it to be Verticillium albo-atrum Reinke and Berthold. Dr. W. L. Gordon of the Dominion laboratory of plant pathology at Winnipeg, identified the Fusarium sp.as Fusarium avenaceum (Fr.) Sacc. The culture of Sclerotinia sp. was identified by Dr. F. L. Drayton, plant pathologist at the Central Experimental Farm, Ottawa, as Sclerotinia trifoliorum Erikss. All three organisms were grown on potato dextrose agar at temperatures ranging from 20° to 23°C.

Verticillium albo-atrum Reinke and Berthold is in the Moniliaceae, Moniliales, of the Fungi Imperfecti. Carpenter (1918), working with the fungus on okra, described it as having ellipsoidal, unicellular

conidia, 4.0 - 11.0 x 1.7 - 4.2μ, abscissed singly from the tips of verticillate-branched conidiophores. The branches of the conidiophores may be from 1 to 7 in a whorl, usually 3 to 5; they may bear secondary branches in virtels, which occur from 30 to 38μ apart along the conidiophores. The terminal branch is usually 1 to 3 times as long as the virtel branches. The mycelium is first hyaline, later becoming brownish; it is septate, and from 2 to 4μ in diameter. Swollen cells resembling chlamydospores, or sclerotia, may occur, but this is a function of the strain. Chaudhuri (1923), working with the fungus in pure culture, found that the conidia averaged 6.8 x 2.5 - 5μ. Bewley (1922), observed that on certain media, particularly those containing asparagin, the fungus from tomato produced slimy salmon-pink spore masses 2 x 1 millimetres, resembling the pseudopionnotes of Fusaria.

In the experiments conducted at Macdonald College, 6 different isolates were used throughout. They were obtained from different plants at various times during the first part of this study, and parallel cultures were maintained. It was thought that strain differences might show up, but these were not observed. The effect of using 6 different cultures in this way was to provide numerous replicates in all the experiments performed. The clover strain of Verticillium albo-atrum conformed in most characteristics with the description by Carpenter, save that no brown or darkened resting hyphae were seen in culture at any time; the mycelium remained hyaline for the duration of these studies.

Conidia of Verticillium albo-atrum from clover, grown on potato dextrose agar, were found to measure from 4.8 - 8.16 x 2.88 - 4.0 μ, averaging 6.19 x 3.40 μ. All measurements were made using the negative nigrosin method for mounting small spores, as recommended by Riker and Riker (1936). A small drop of spore suspension was placed on a clean slide, nigrosin added, and the spores mixed with the nigrosin, then spread in a thin film on the slide. When dry, balsam and a cover slip were added. The spores appeared light against a dark background, making the measurements much easier than if they were mounted in a light-passing medium.

Spore masses as described by Bewley (1922) were not observed in the fungus on potato dextrose agar, but after about two weeks grown on slants, the mycelium which at first is white and fluffy, seems to dry down to a certain extent, and a yellow, dry, crust-like aggregation of spores forms about the centre of the culture, or along the line where the needle was streaked. Great numbers of spores are produced all over the surface of the culture; aggregations of spores give the colony a typical granular appearance. The medium, particularly in slants, assumes a lemon-yellow color by reflected light; by transmitted light, the yellow seems to have a greenish tinge.

Fusarium avenaceum (Fr.) Sacc. is in the Tuberculariaceae, Moniliales, of the Fungi Imperfecti. A complete description of the fungus isolated from cereals in England, and grown on various media, is given by Bennett (1928), who states that the aerial mycelium on potato dextrose agar is abundant, fluffy, white, with transient traces of rose and rellow. Medium: on potato dextrose agar the plectenchyma

is yellowish, then carmine, then ox-blood red, with the deeper layers eventually turning to shades of brown. Sporodochia on this medium are few and small. Microconidia occur; the 3 -septate ones are predominant. These are spindle-shaped, and from 23 - 27.5 x 4.3 - 5.5μ. Non-septate ones, and 1 -septate ones, are also found. Non-septate microconidia are elliptic, oblong, or fusoid, 11.6 - 20.3 x 3.0 - 3.5μ. The macroconidia are slender, elliptically curved; they may be straight for the greater part of their length, then curve sharply at the upper end. They are nearly uniform in width throughout, become narrow gradually at both ends. Walls and septa are very thin. Macroconidia are borne on the hyphae of the aerial mycelium, in sporodochia, and pionnotes. The 5-septate macroconidia predominate, occurring in up to 95% of the cases. From potato dextrose agar, those in sporodochia measure 54.5 x 4.0μ; those on the mycelium 57.0 x 3.5μ(vary from 43.1 - 65.9 x 3.5 - 3.6μ).

The strain of Fusarium avenaceum found associated with Sclerotinia trifoliorum on wilted clover at Macdonald College agrees with the above description in regard to coloration. So few spores have been produced, however, that no information on their size or other characteristics is available. In general appearance on potato dextrose agar slants, this strain is indistinguishable from one isolated from clover by M. W. Cormack at Edmonton, Alta; a culture of his strain was available for comparison.

Sclerotinia trifoliorum Erikss is in the Helotiaceae, Pezizales, of the Ascomycetes. The mycelium is coarse, grayish, and on potato dextrose agar slants and plates forms a very sparse growth over the

surface. Wolf and Cromwell (1919) refer to the attachments which the fungus forms in culture. The hyphae become flattened, septate, profusely branched and interlaced, resulting in the formation of dark masses which adhere to the glass surfaces. The same authors refer to microconidia which may be formed. These are small, spherical, 2 - 4 μ in diameter, abstricted from flask-shaped cells; they may form in conspicuous, grayish, mealy patches on the surface of the mycelium in culture. They are not known to have any function as yet; inoculations with them failed in all cases. Sclerotial formation in culture is also described. Sclerotia begin as dense, floccose, white mycelial masses; after a few days these masses have increased in size, become more compact, and cartilaginous in consistency. At first they are white in color, but the exterior rapidly becomes inky black; the internal tissue remains white. Mature sclerotia are hard and leathery.

The attachments on glass surfaces described above have been observed in the cultures of Sclerotinia trifoliorum studied during this project. Some microconidia have also been found, but they were always few in number. It has been observed repeatedly that during the process of sclerotial formation, drops of liquid are present on the surface of the mycelial masses. Sclerotia form at ordinary temperatures in about ten days; in plates, they characteristically form a ring around the outer edge of the plate, as illustrated in Fig. 7. The sclerotium in the centre of the plate is the one used as inoculum.

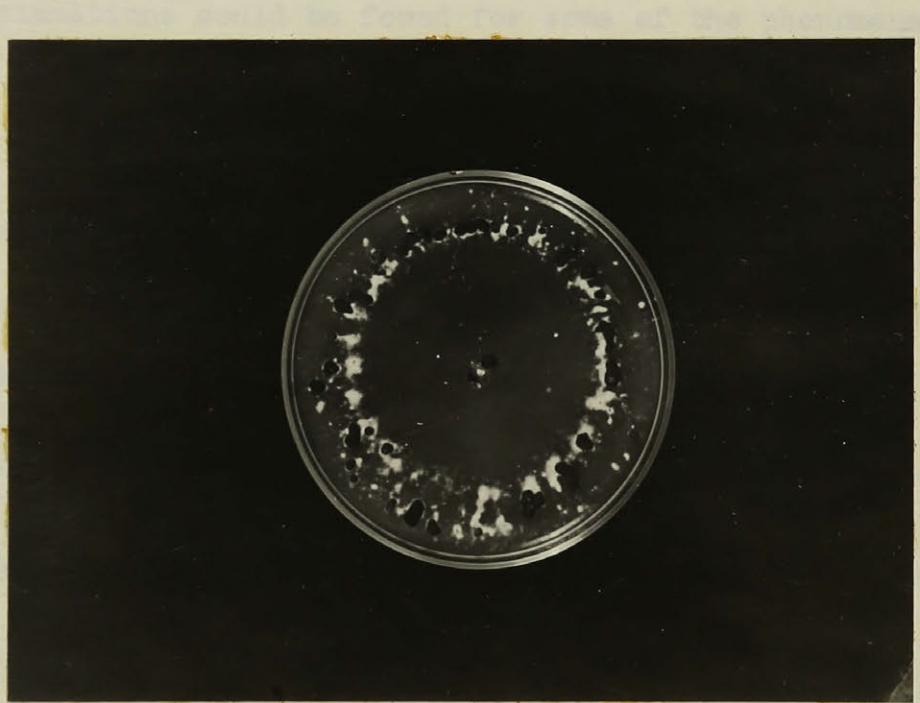

Fig. 7. Sclerotinia trifoliorum in pure culture, showing typical formation of sclerotia in a ring near the outer edge of the plate.

Physiological Studies.

Certain tests were conducted on all 3 fungi in pure culture, to learn something of their physiology. It was hoped that by this means explanations could be found for some of the phenomena observed in field studies.

Temperature Relationships.

Rudolph (1931) gives the following table, showing the temperature relations of Verticillium albo-atrum in pure culture, as reported by various investigators.

Table 3 - temperature relationships of Verticillium spp.

Author	Species	Degrees centigrade.		
		Minimum	Optimum	Maximum
Bewley	V. albo-atrum	4.4	23.3	30
Potschke	V. albo-atrum	5-10	28	33-37
Czarnecki	V. species	10-14	20-25	35-37
Chaudhuri	V. albo-atrum	10	22.5	27
Edson and Shapovalov.	V. albo-atrum	5	25	30-35

Rudolph is of the opinion that the optimum found by Potschke, at 28°C is an error, as it is so much higher than the other reports. Verona and Ceccarelli (1935), comparing 4 species of Verticillium, including V. albo-atrum, found that all species made their best growth at 24°- 26°C. Ludbrook (1933) reported the upper limits for growth of V. albo-atrum to lie between 28°-30°C. Ayers and Hurst (1939) found the cardinal temperatures for V. albo-atrum on potato dextrose agar to

be 4°C, 19°- 21°C, and 30°- 32°C. Chaudhuri (1923) used a series of different media in his tests with this fungus, and found that, although the maximum temperature for growth varied with the food material, the optimum remained the same. His strain of V. albo-atrum did not show any growth after a week at 33°C; when placed at 22.5°C, the temperature he found to be optimum, the fungus failed to grow. At 5°C it did not grow in the course of a week, but when transferred to 22.5°C, it grew well. This is in direct contrast to the findings of Sarajanni (1938). He states that a strain of V. albo-atrum, isolated from wilted cotton in Greece, withstood temperatures exceeding 40°C and below 0°C. The viability of his organism was such that it grew after 3 years in the laboratory without subculturing.

To determine the temperature relationships of the V. albo-atrum strain from clover, the fungus was grown on potato dextrose agar in petri plates, 15 cc. per plate. A drop of spore suspension was placed in the centre of each plate, and the cultures were incubated in temperature chambers maintained at 4°, 8°, 12°, 16°, 20°, 24°, 28°, 32°, and 36°C. respectively. Three plates of each strain were used in each chamber, giving 18 plates at each temperature. The diameter of the colonies was measured in millimetres every 12 hours for 10 days. The results of these measurements are given in Table 4.

It can be seen from these figures that the fungus did not grow at 4°C, nor at 36°C. The optimum temperature for growth was at 28°C. This information is presented graphically in Fig. 8. From the figure it may be seen that, although the optimum for growth was at 28°C, growth was almost as good at 24°C. As the growth rate does not drop

Table 4. Diameter of colonies (in millimetres) of Verticillium albo-atrum grown on potato dextrose agar. Measured every 12 hours for 10 days.

Temperature (centigrade) DAYS--	1½	2	2½	3	3½	4	4½	5	5½	6	6½	7	7½	8	8½	9	9½	10
4°	no growth																	
8°	--	--	1	1	1	1	1	1	2	2	2	2	2	2	3	3	4	5
12°	--	1	1	2	3	4	5	6	7	8	9	10	11	11	12	14	14	16
16°	--	1	3	5	7	8	10	11	13	15	16	17	19	22	23	25	26	28
20°	1	4	6	8	11	13	16	19	21	24	26	29	32	34	37	39	42	45
24°	2	6	9	12	16	19	22	26	29	32	36	39	43	46	50	53	56	59
28°	4	7	11	14	18	21	25	28	32	35	39	43	46	49	53	56	60	63
32°	2	4	6	8	11	14	17	20	22	24	27	29	32	35	38	40	43	46
36°	no growth																	

before the fungus reaches the edge of the plate, it is evident that no staling occurs on the medium used at any of the temperatures maintained. The uniform increase in diameter from day to day is shown in the graph in Fig. 9. From this graph can also be seen that the optimum temperature remained the same throughout the course of the experiment. The rapid fall of the curve above the optimum temperature shows how much closer the optimum is to the maximum temperature than to the lower temperatures at which the fungus will grow. No growth was seen at either 4°C or at 36°C at the end of a week. Plates from both these temperatures were placed at 28°C. The cultures from 4°C grew vigorously; those from 36°C made no growth at all.

The measurements taken were the diameters of the colonies; no expression was found for the type of growth of the fungus. Examination of the plates at the conclusion of the experiment showed that the mycelial growth at temperatures of 20°C and below was much denser than in the warmer chambers. The mycelium in plates incubated at 24°C and 28°C was closely appressed to the medium, and growth was much thinner than at the lower temperatures. It was observed also that the characteristic yellow pigmentation of the medium increased in intensity from 8° to 24°C; at 28°C, it was observed in some plates, but not in all of them; and it was not seen at 32°C.

Fusarium avenaceum was found by De Haan (1937) to have as its cardinal temperatures in modified Richard's solution 3°C, 18°-27°C, and 33°C. Its temperature relationships were similar on a malt agar medium. Cormack (1937) found that F. Avenaceum from alfalfa and sweet clover roots had its minimum at 1°C, its optimum at 24°C, and its maximum at 34°C. Tu (1930) studied 2 forms of F. avenaceum

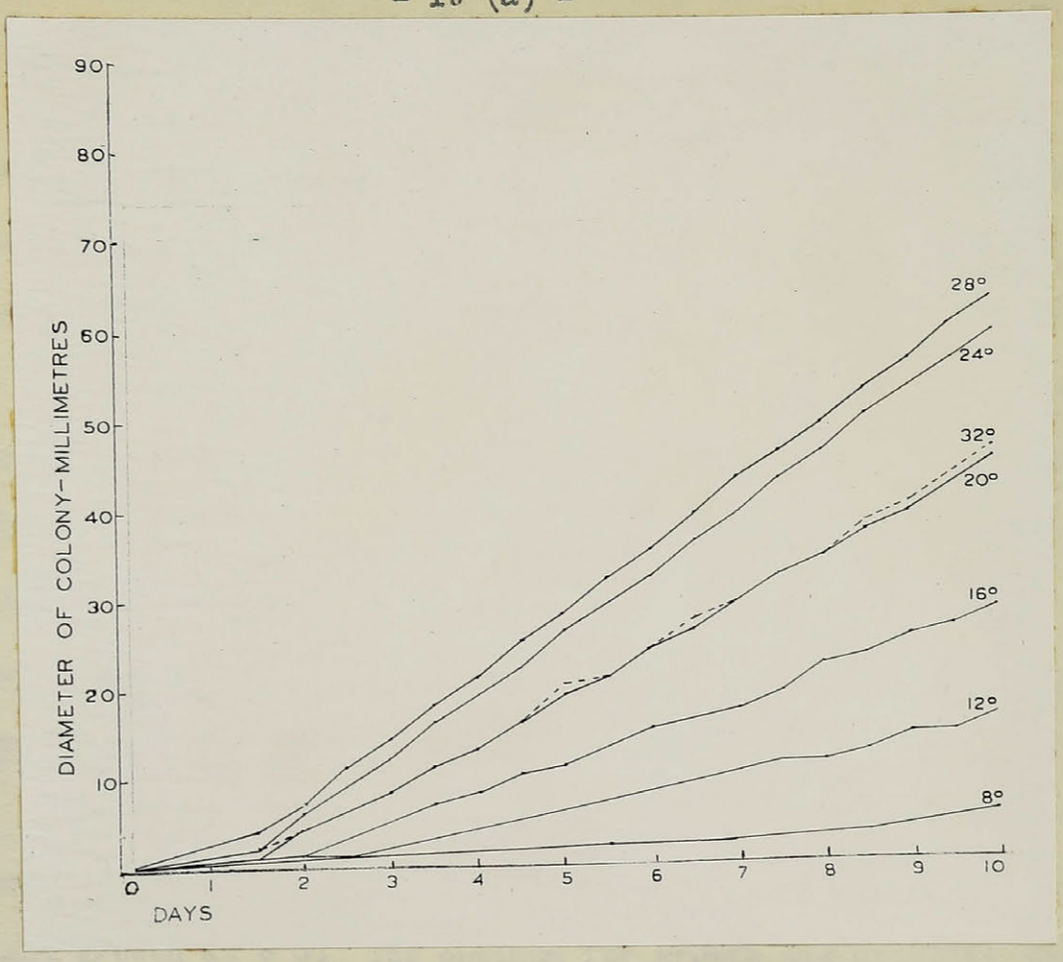

Fig. 8. The effect of temperature on Verticillium albo-atrum

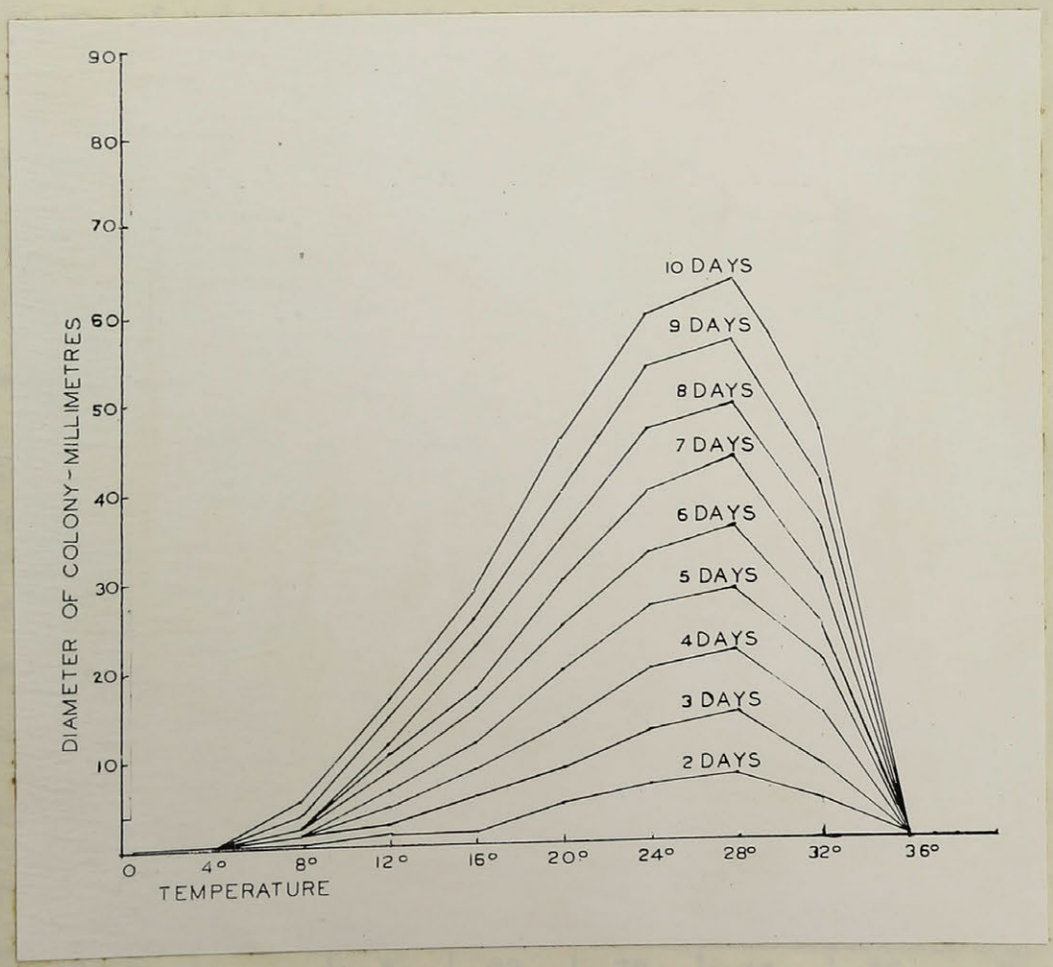

Fig. 9. Daily growth of Verticillium albo-atrum.

causing headblight of cereals. He grew his fungi on 18 cc. of potato dextrose agar in petri plates, at temperatures from 7° to 37°C, with 5°C intervals. Form 1 had its optimum at 22°C, form 2 at 27°C. For most the species of Fusarium studied by Tu, the optimum temperature was 27°C. Fusarium nivale, however, also had its optimum at 22°C, and did not grow at all at 32°C. Starr (1932), working with 5 cultures of Fusarium spp. causing pea wilt, found the optimum temperature for 4 of them to be 28°C, while the optimum for Fusarium acuminatum was 20°C.

The strain of Fusarium avenaceum found associated with red clover plants at Macdonald College showed no growth at 4°C, but slight growth at 8°C. Its optimum for growth was at 20°C. Growth occurred at 28°C, but none was seen at 32°C. The fungus was grown on 15 cc. of potato dextrose agar in petri plates, inoculated at the centre with a small loopful of a water suspension of mycelium. Three plates were used in each temperature chamber, kept at 4°, 8°, 12°, 16°, 20°, 24°, 28°, and 32°C, as with Verticillium albo-atrum. Readings were taken every day for 8 days. The results are tabulated below.

Table 5. Diameter of colonies (in millimetres) of F. avenaceum.

Temperature (centigrade)	Days-- 1	2	3	4	5	6	7	8½
4°	----------			no growth				----------
8°	--	--	--	--	--	--	--	10
12°	--	---	---	11	16	26	32	43
16°	--	--	6	20	29	39	47	63
20°	--	3	17	33	45	59	68	86
24°	--	5	22	37	44	52	56	64
28°	--	11	23	29	33	39	42	49
32°	----------			no growth				----------

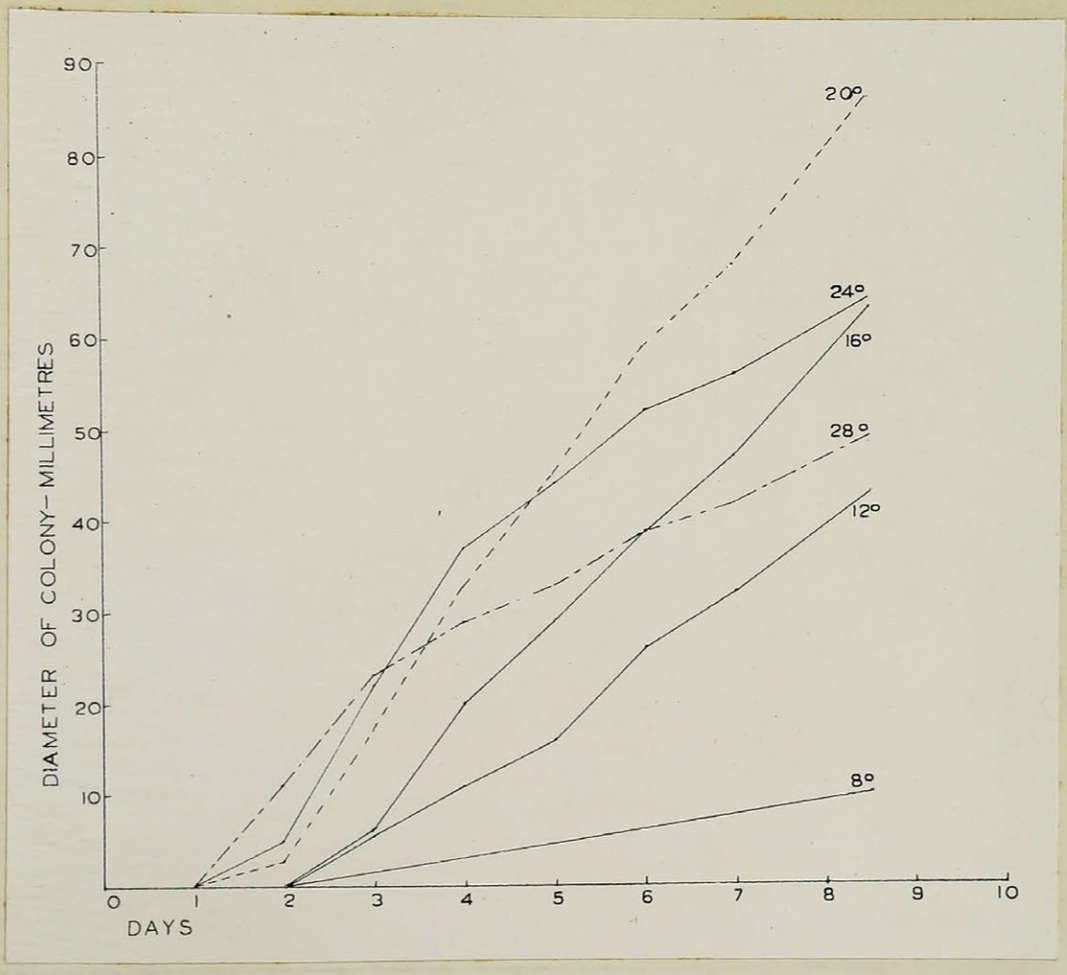

Fig. 10. The effect of temperature on Fusarium avenaceum.

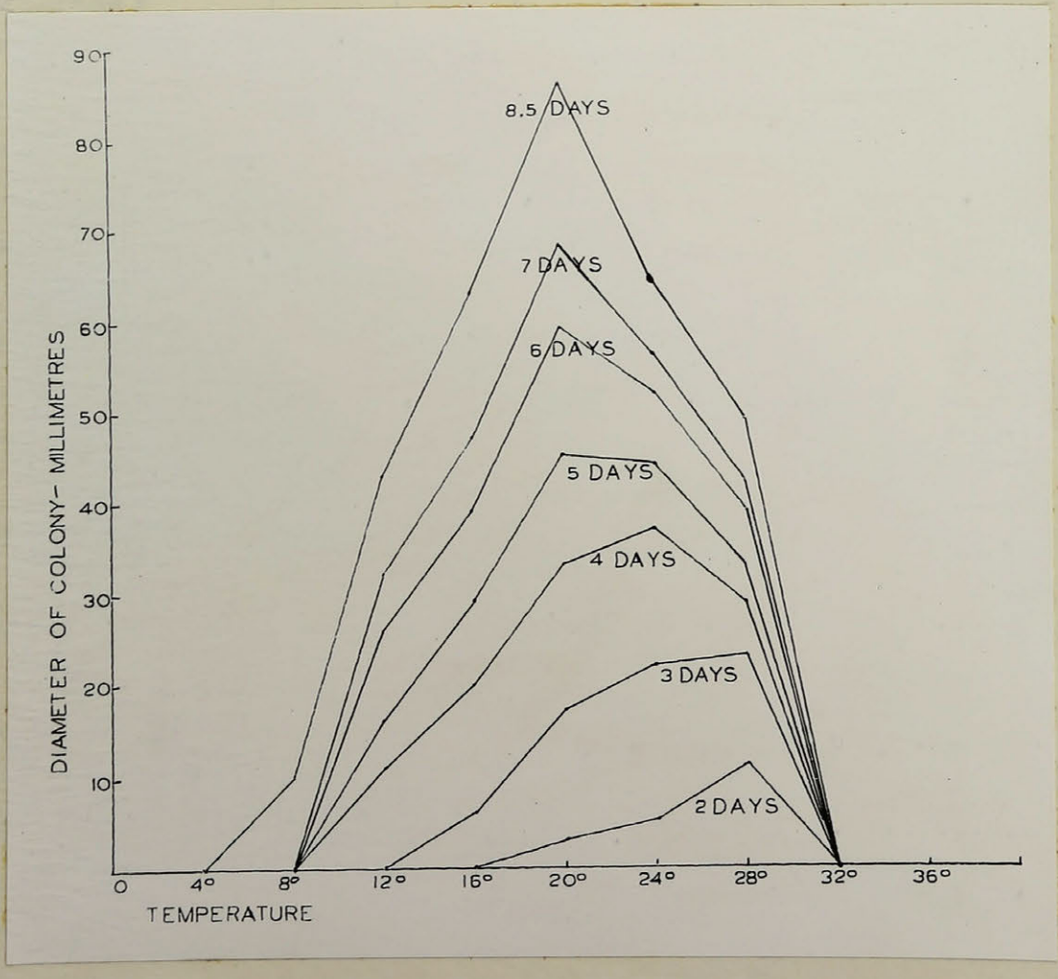

Fig. 11. Daily growth of Fusarium avenaceum.

with growth good from 13° to 26°C.

Temperature relations for the strain of Sclerotinia trifoliorum used in the experiments here reported, were found by using the same methods already outlined. The plates were inoculated with a single sclerotium instead of a mycelial suspension, however, as this was found to be the more satisfactory method. The mycelium of this organism spread very rapidly over the surface of the agar, so that the plates were covered in 5 days at the optimum temperature. Very little mycelium was produced, however; the mat was so thin that some difficulty was experienced at first in making the measurements. The results are tabulated below:

Table 6. Diameter of colonies (in millimetres) of S. trifoliorum.

Temperature (centigrade)	Days— 1	2	3	4	5	6	7	8½
0°	----							
4°	--	--	--	--	8	12	16	23
8°	--	--	7	14	20	32	43	69
12°	--	--	10	27	40	65	82	90
16°	--	9	22	48	73	90		
20°	--	9	28	60	90			
24°	--	11	26	44	64	82	90	
28°	--	0.5	1	1.5	2	2	2	2
32°	----							

The table, and growth curves in Fig. 12. show the optimum temperature for growth to be 20°C. No growth was seen at 0°C; very little growth occurred at 28°C, and none at 32°C. The growth curves for this fungus are very much steeper than those for the other 2 organisms studied; although its optimum and maximum temperatures are similar to those of Fusarium avenaceum, it makes vigorous growth at much lower temperatures. Fig.13 shows that this fungus, like F. avenaceum, undergoes a shift in the optimum temperature. Growth is seen to fall off from the optimum at the higher temperatures even more sharply than it does with F. avenaceum.

pH Relationships.

The pH relationships of all 3 organisms were determined by the following method. Regularly increasing quantities of sterilized half-normal sodium hydroxide were added aseptically to 10 cc. quantities of hot sterile potato dextrose agar, which was then poured into plates. The acid range was obtained by using sterilized half-normal hydrochloric acid, added in the same way. The pH levels thus obtained were determined by means of a glass electrode potentiometer. The levels used were pH 2.65, 3.2, 4.25 (obtained by adding acid), pH 5.4 (the unaltered medium), and pH 6.1, 7.2, 7.9, 8.5, and 9.15 obtained by adding alkali. Three plates were used for each fungus at each pH level. All plates were left on the laboratory bench for 7 days, at temperatures ranging from 22°to 25°C.

Guba (1934) reported that Verticillium albo-atrum in culture was inhibited at pH 4.0, and made its optimum growth at pH 6.0 - 7.0.

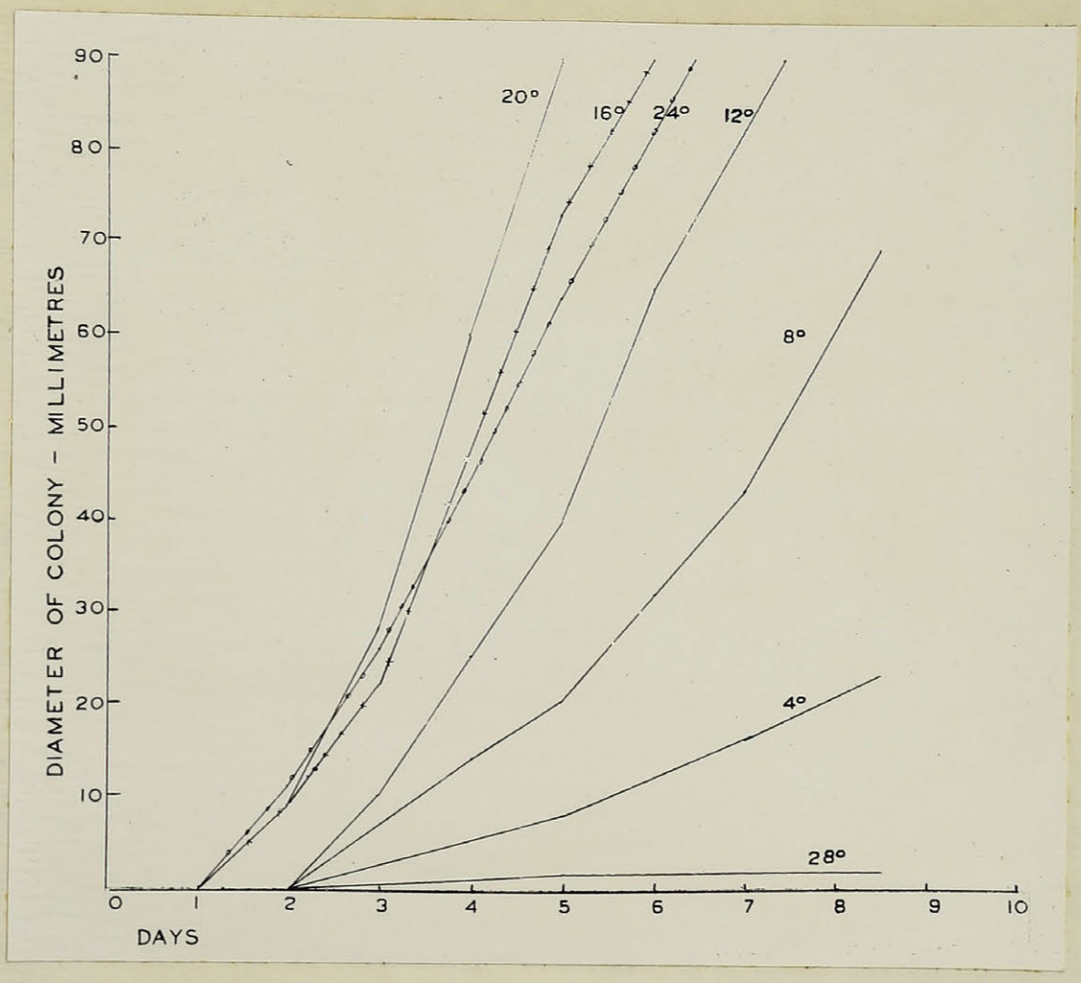

Fig. 12. The effect of temperature on Sclerotinia trifoliorum.

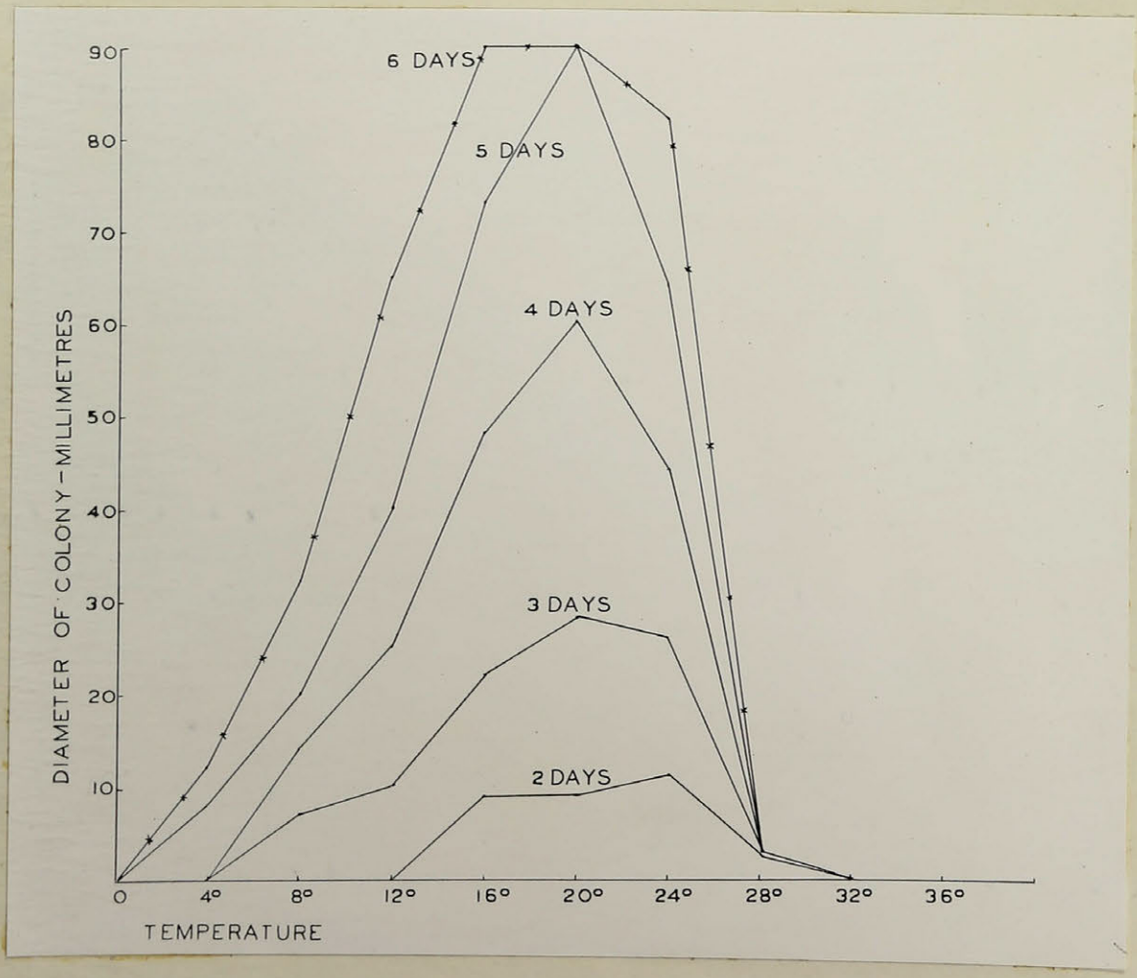

Fig. 13. Daily growth of Sclerotinia trifoliorum.

Verona and Ceccarelli (1935) studied the pH relationships of 4 species of Verticillium. The optima they found were, for V. albo-atrum, pH 8.5; V. dahliae, pH 4.9; V. tracheiphilum, pH 5.6; and for V. amaranti, pH 5.0.

The results obtained with the red clover strain of V. albo-atrum are tabulated below. From the figures given, it can be seen that

Table 7. Diameter of colonies (in millimetres) of V. albo-atrum.

pH 2.65	pH 3.2	pH 4.25	pH 5.4	pH 6.1	pH 7.2	pH 7.9	pH 8.5	pH 9.15
9	19	38	38	39	39	35	35	31

the fungus made good growth throughout the range pH 4.25 to pH 9.15; the differences between plates within this range are so slight that no significance can be attached to them. Growth at pH 3.2 and 2.65 was much less vigorous.

Fusarium spp. have been reported to grow over a very wide pH range. Mix and Vaughn (1924) found that F. tracheiphilum from cowpea, grew on potato dextrose agar over a range of pH 2.4 to pH 12.2. Cormack (1937) found that the optimum pH for F. avenaceum was 5.8 and 7.7; less growth was seen at pH 6.2. The fungus grew at all pH from 2.8 to 9.5.

The results obtained for F. avenaceum in this study are given in the following table. The results conform with those reported in the literature, in respect to the range of pH at which the fungus will grow. The optima at pH 5.4 and 6.1, however, are lower than those given by Cormack, and no intermediate isoelectric point was observed.

Table 8. Diameter of colonies (in millimetres) of F. avenaceum.

pH 2.65	pH 3.2	pH 4.25	pH 5.4	pH 6.1	pH 7.2	pH 7.9	pH 8.5	pH 9.15
6	17	39	58	58	45	42	37	26

Pape (1937) found that Sclerotinia trifoliorum would tolerate a wide range of hydrogen ion concentrations. It grew best on the acid side of the scale, at about pH 5.5. This value is somewhat lower than the one found to be optimum in the experiment, the results of which are given below; but the fungus is seen to tolerate acid conditions much better than alkaline ones, as Pape indicated.

Table 9. Diameter of colonies (in millimetres) of S. trifoliorum.

pH 2.65	pH 3.2	pH 4.25	pH 5.4	pH 6.1	pH 7.2	pH 7.9	pH 8.5	pH 9.15
40	47	47	46	65	80	47	14	no growth

Effect of light on Verticillium albo-atrum.

During some inoculation work with V. albo-atrum in the greenhouse, it was observed that test-tube cultures of the organism changed in color from their normal white to salmon pink. No reference to a similar phenomenon could be found in the literature. Most investigators who study the effect of light upon fungi report the effect on reproduction and growth rate; Rabinovitz-Sereni (1932) found that, in 9 out of 10 organisms he studied, white daylight and incandescent electric light stimulated mycelial growth, sporulation, and the formation of pigment. Other workers have found that the pigments normally occurring in the fungus may be intensified in daylight, but no report has been seen of the production in light of

a pigment not normally seen in the fungus.

Experiments were conducted to determine some of the conditions affecting this pigment production, and to find if the color is constant once acquired. Cultures were grown in test-tubes and 125 cc. Ehrlenmeyer flasks, using both potato dextrose agar and Czapek's agar. Tubes were used in most of the experiments; when they were repeated, flasks were used. Transfers were made from cultures grown in an incubator, to potato dextrose agar slants. Six slants were exposed to the light in a greenhouse painted white, 6 were left on the bench in a basement laboratory where daylight was very poor, and bright electric illumination was used; 6 were placed in a dark cupboard in the same laboratory, and 6 were replaced in the incubator, in which a very dim yellow light bulb burned constantly. The cultures exposed to greenhouse conditions all turned salmon pink within 3 days; all of the others developed as ordinary white mycelium. The experiment was repeated using Czapek's agar in flasks, with the same results. To find what intensity of light caused the coloration, the experiment was modified slightly. Potato dextrose agar plate cultures were used. In addition to the places previously used, groups of 6 plates were also placed on the window sill, on a bench several feet from a window, and on a table in the centre of a light, sunny laboratory. It was found that the coloration developed most rapidly, and was most intense, in the plates on the window sill; those near the window showed more color than the ones in the centre of the room. This effect was not due to temperature, because cultures were kept in a cool greenhouse during February, and at

various temperatures in an incubator. The cultures in the greenhouse became colored, while the others did not. Cultures in plates kept covered in a greenhouse developed no coloration.

Transfers were made from the pigmented mycelium; one series was placed in an incubator, one left in the dark laboratory, and another placed in the greenhouse. The greenhouse cultures developed the salmon-pink color; those in the laboratory and incubator grew normally, showing that the character was not transmissible when the stimulus was removed. Colored cultures were placed in the dark for 2 week intervals, but they retained their color.

This effect of sunlight on V. albo-atrum explains the occurrence, on clover tissues in the field, of pink as well as white mycelial masses. The pink fungous growth was in all other respects identical with the normal V. albo-atrum, and characteristic cultures of the organism were isolated from it repeatedly. The pink growth occurred on stems and crown tissues exposed to the light.

Filtrate Studies.

Rudolph (1931) in his study of Verticillium hadromycosis, reviewed the literature on the cause of wilting induced by Verticillium. One group of authors supported the thrombosis theory, which assumes that wilt is due to the plugging of the vessels by mycelium, gum-like substances, tyloses, or other materials. Little experimental work has been done in support of this theory; thrombosis has been assumed, rather than proved, to be the cause of wilt. Another group of investigators believe that wilting is due to the production of toxic materials by the fungus within the host; considerable experimental evidence has

been brought forward to support this theory.

Bewley (1922) working on Verticillium wilt of tomato, grew V. albo-atrum for 30 days on Dox's medium. Tomato seedlings, cut off near the roots under water, were placed in carefully filtered medium in which the fungus had grown. Filtrate which had been heated to 100°C for 5 minutes was also used. Plants in uninoculated medium and water were used as checks. Rapid wilting occurred in plants in the filtrate; heating did not prevent wilting, showing that wilt was not due to an enzyme. The check plants were unaffected.

Linford (1931) used a similar method to test a species of Fusarium which caused wilt of peas. The pea seedlings were tested in the filtrate from Richard's medium in which the fungus had grown for 15 - 22 days. He diluted some of his filtrate and sterile check medium to half strength and quarter strength with distilled water. Plants wilted more slowly in the diluted filtrate than in the concentrated material; check plants in sterile medium and in water did not wilt.

Clover plants were used in similar filtrate studies with Verticillium albo-atrum, Sclerotinia trifoliorum, and Fusarium avenaceum. One such study was made, using V. albo-atrum grown on Richard's medium for 30 days at 23°C. The medium was carefully filtered, first through cheesecloth to remove large particles, then through a Seitz filter. The filtrate was used at full strength and at half strength, diluted with distilled water. Uninoculated medium at full strength and half strength, and distilled water, were used as checks. Leaves were cut from young plants of red clover under water; the lower end of the petiole was removed with a razor blade, under water, and the leaf placed in the test solution in small test

tubes. All tests were run in triplicate. The results at the end of 12 hours are tabulated below:

Table 10. Clover leaves in filtrate from V. albo-atrum 12 hours.

Test	No. of tubes	Results (effect on leaves)
1. Filtrate 100%	18	All wilted.
2. Filtrate 50%	18	17 wilted; 1 unaffected.
3. Sterile medium 100% (check)	3	Unaffected.
4. Sterile medium 50% (check)	3	Unaffected.
5. Water (check)	6	Unaffected.

Wilting was gradual; the leaves first became flaccid, then gradually dried out. In the course of wilting, the leaflets drooped, and the petiole bent downwards. The photograph in Fig. 14. shows the final stage of wilt. This photograph was taken of material from a preliminary experiment in which stems with several leaves, as well as single leaves, were used. It was found that the single leaves were easier to handle and gave the same results as did whole young plants, so they were used in all subsequent filtrate experiments.

A more elaborate filtrate experiment was performed, to test the effects of Fusarium avenaceum and Sclerotinia trifoliorum as well as Verticillium albo-atrum. The fungi were grown on Richard's medium for 14 days at temperatures ranging from 23° to 27°C. V. albo-atrum and F. avenaceum produced thick surface mats of mycelium in this period; S. trifoliorum showed only a slight growth of submerged mycelium. The respective filtrates were prepared by the method reported above. In addition to the test at half strength,

Fig. 14. Tubes in "1" contain full strength and half strength filtrate, in "2" full strength and half strength sterile medium, and in "3" distilled water. Photographed 24 hours after start of test.

the filtrates were also tried at quarter strength, diluted with distilled water as before. Half the filtrate from each fungus was autoclaved 20 minutes at 15 pounds pressure to try the effect of heat. Tests on F. avenaceum and S. trifoliorum were run in triplicate; 6 tubes were used for each test with V. albo-atrum. Sterile medium at the 3 strengths and distilled water, were used as checks. Clover leaf material was prepared as before. The results are given in Table 11. The readings tabulated were made after 24 hours; no changes had been observed in readings after 6 and 12 hours. Readings at 48 hours showed no further change had occurred.

It is believed that the results are less conclusive than those of the previous experiment because of the shorter interval in which the fungi were grown in the medium, thus cutting down the accumulation of toxins. The possible effect of time was not realized until the results for V. albo-atrum filtrate were compared with those obtained previously, in which the same type of medium and clover plants at about the same stage of development were used.

Table 11. Clover leaves in fungous filtrates 24 hours.

Test	No. of Tubes.	Results
Verticillium.		
Filtrate 100 %	6	2 unaffected; 2 turgid, but leaf margins rolled downward and are discolored as though scalded; 2 wilted.
Filtrate 50%	6	2 flaccid; 1 flaccid, leaf margin 'scalded' 3 wilted.
Filtrate 25%	6	Unaffected.
Fusarium.		
Filtrate 100%	3	2 wilted; 1 unaffected.
Filtrate 50%	3	3 unnaturally hard, brittle.
Filtrate 25%	3	1 flaccid; 2 unaffected.
Sclerotinia.		
Filtrate 100%	3	1 flaccid, margin of leaf "scalded"; 2 turgid, margins of leaves "scalded".
Filtrate 50%	3	1 flaccid; 1 turgid, margin of leaf "scalded"; 1 unaffected.
Filtrate 25%	3	1 flaccid; 2 unaffected.
Filtrates autoclaved 20 minutes at 15 lbs. pressure.		
Verticillium		
Filtrate 100%	6	1 flaccid, "scalded"; 2 turgid, but "scalded" around margins; 3 wilted.
Filtrate 50%	6	5 unaffected; 1 wilted.
Filtrate 25%	6	4 unaffected; 2 flaccid.
Fusarium		
Filtrate 100%	3	2 hard, brittle, margin "scalded"; 1 wilted;
Filtrate 50%	3	1 wilted; 1 flaccid; 1 unaffected.
Filtrate 25%	3	1 flaccid; 2 unaffected.
Sclerotinia.		
Filtrate 100%	3	1 wilted; 2 unaffected.
Filtrate 50%	3	3 unaffected.
Filtrate 25%	3	3 unaffected.
Sterile Medium(check)		
100%	3	Unaffected.
50%	3	Unaffected.
25%	3	Unaffected.
Distilled water (check)	6	Unaffected.

Inoculations on aseptic seedlings.

An interesting method used in studying the pathogenicity of fungi, is inoculating them onto host plants which have been grown under aseptic conditions. Bewley (1922) grew tomato seedlings aseptically in studying Verticillium wilt of that plant. He surface sterilized tomato seeds in mercuric chloride, washed them in sterile water, and transferred them onto agar in petri dishes to germinate. The seedlings were then transferred to 200 cc. of Czapek's agar in 1 litre Ehrlenmeyer flasks. After 5 days growth, pure cultures of V. albo-atrum were inoculated onto the agar in the flasks, and the plants observed periodically for the appearance of wilt. Fergus and Valleau (1926) used a different method with red clover. They surface sterilized the seed in formalin, 1:250, for 25 minutes at 40°C. The seeds were germinated on agar in petri dishes; when germination was well started, the seedlings were transferred to 10 cc. of nutrient agar in test tubes. Organisms to be tested were inoculated onto the surface of the agar.

The method used in these studies was an adaptation of the one described by Bewley. His method was used without alteration in the first series of experiments, but the use of large flasks and large quantities of agar did not lend itself to extensive tests. For that reason, 50 cc. of Czapek's agar was used in 125 cc. Ehrlenmeyer flasks, and this proved quite satisfactory. The seed was surface sterilized by flooding it with 95% alcohol in a sterile petri plate. The plate was then inverted at an angle, and as the alcohol evaporated and the seeds dried, they fell to the lower

edge of the plate. This method obviated the need of rinsing the seeds in water, and was satisfactory from an efficiency standpoint; it was recommended by Dr. P. H. H. Gray of the Bacteriology Department. Attempts were made to eliminate the agar plates for seed germination, by placing surface sterile seeds directly on nutrient agar in the flasks. It was found, however, that germination was so poor on the nutrient agar, that to ensure a plentiful supply of seedlings, and avoid waste of time and materials, germination was best carried out on clear agar in petri plates stored 48 hours at 22°C, under bell jars to preserve a saturated atmosphere. Vigorous uncontaminated seedlings were selected from the germination plates, and transplanted 2 to a flask. A period of several days was allowed for the plants to become established before the test organism was inoculated onto the agar. Inoculations were made near each plant.

In one experiment, 6 flasks inoculated with V. albo-atrum were kept in a dark basement laboratory, 6 flasks were placed in a bright sunny laboratory, and 12 flasks were placed in the greenhouse. Check flasks which had not been inoculated were included with each test. Readings taken after 12 days showed all plants in the check flasks to be healthy. In the inoculated flasks in all 3 tests, all the plants were dead but 1 in the greenhouse. In this flask the fungus did not grow toward the plant, showing that contact with the host is required before toxic effects can occur.

In another experiment, inoculations were made as follows; 6 flasks with V. albo-atrum; 6 flasks with V. albo-atrum and Sclerotinia trifoliorum; 6 flasks with S. trifoliorum; 6 flasks with S. trifoliorum and Fusarium avenaceum; 6 flasks with F. avenaceum; 6 check flasks, not inoculated. This test was run in the basement laboratory, with results as given below at the end of 5 days. All the inoculated plants were dead and drying out by 15 days.

Table 12. Inoculations on aseptic clover seedlings.

Treatment.	Results (5 days after inoculation)
V. albo-atrum.	6 plants wilted, 6 erect but roots discolored.
Vert. & S. trifoliorum.	10 wilted, 2 erect.
S. trifoliorum.	9 wilted, 3 healthy (erect)
Scler. & F. avenaceum.	11 wilted, 1 erect.
F. avenaceum.	11 wilted, 1 erect.
Check.	12 healthy.

The "wilted" classification in the above table includes all plants which were dead, those which actually appeared wilted, and those which though not appearing badly diseased, were affected so that the stems were not sufficiently turgid to support the leaves, causing the seedling to fall against the side of the flask.

The symptoms developed by seedlings incoculated with any of the 3 fungi studied, or combinations of them, were essentially similar.

Fungous attack took place in most cases at the surface of the agar, causing a browning and constriction of the stem of the seedling, which fell over. The top died gradually as a rule. In some cases the roots were overgrown by the fungus before any appreciable injury to the plant appeared, as with Verticillium, or, to a lesser extent, with Fusarium. Sclerotinia, however, at no time developed more than a few hyphal threads, forming a very thin network on the surface of the agar; some seedlings in flasks inoculated with Sclerotinia seemed to fall over before the mycelium reached them. In one test, where the seedlings were allowed to develop 5 leaves before being inoculated with V. albo-atrum (in 500 cc. flasks), the plants were not killed. Brown lesions developed on the roots, and inoculated plants appeared much less vigorous than the check plants, but they seemed able to withstand the fungus once they had passed a certain stage; as some roots were killed, new ones were formed, and adventitious roots were observed to form along the basal $\frac{1}{2}$ inch of the stem.

Seed Studies.

Reports by various investigators on the occurrence of Sclerotinia trifoliorum in samples of red clover seed, and the isolation of Fusarium spp. from pea and clover seeds led to the belief that clover seeds in Quebec might be infested with these organisms. Thirty samples of red clover seeds from various parts of the province were used in isolation studies. These samples had

been harvested over the period 1927 to 1939. Germination studies were run on all samples by the Seed Testing Laboratory in Montreal, in the hope that germination might be correlated with the occurrence of fungi in the seeds. Surface sterilized seeds were planted on potato dextrose agar and incubated at 22°C. About 1200 seeds were so tested, and only 19 of them showed any fungal contamination. These organisms were common contaminants, and no one species occurred consistently, so it was assumed that seed transmission of organisms pathogenic on red clover was of no importance on the samples studied.

Histological Studies.

Stem and root tissues from clover plants showing either wilt or root rot in the field were preserved in formalin-alcohol-acetic killing and fixing solution, then imbedded in paraffin using the N-butyl-alcohol series as outlined in Riker and Riker (1936). Material from healthy plants, and from rotted inoculated roots, on which Verticillium albo-atrum was present, was also imbedded. Serial sections were cut from 5 to 12 microns thick. The stains used were safranin and fast green, Flemming's triple stain, and carbol-thionin and orange G. No mycelium was seen in the vessels of material from wilted plants; serial sections from one piece of tissue showed 3 of the vessels in the root of a wilted plant to be plugged with a dark-staining material. The material showing V. albo-atrum on the surface was too badly rotted for any details to be distinguished. The vessels of roots and stem bases from

diseased plants showed a brown discoloration in some of the sections; this discoloration was visible to the naked eye in free-hand cross-sections of the same material.

Antagonism Studies.

Observations made during the seedling inoculation studies on Czapek's agar in small flasks showed that when V. albo-atrum and S. trifoliorum were inoculated into the same flask, the dense mycelial growth of V. albo-atrum covered S. trifoliorum, which grew more slowly.

Fusarium avenaceum and Sclerotinia trifoliorum were inoculated onto opposide sides of petri plates containing potato dextrose agar, and incubated at temperatures from 4° to 36°C. At temperatures favorable for the growth of both fungi, they were observed to spread towards each other until their mycelium met. The more rapidly spreading S. trifoliorum grew around the smaller colonies of F. avenaceum. Both fungi appeared to be inhibited along the line of contact. The mycelium of these species was not observed to intermingle, nor did one fungus overgrow the other.

GREENHOUSE STUDIES.

Wilt Symptoms.

Mature clover plants were potted and brought into the greenhouse for study in the fall of 1939. Several months later symptoms of disease began to appear on some of the plants. As plants wilted, they were segregated in an attempt to keep the disease from spreading, and for observation. The symptoms developed in the greenhouse differed from those in the field, in that in many cases one side of a plant would appear dead or diseased, while the rest might seem healthy; in the field, the whole plant commonly showed the effects of disease. In other respects, the symptom picture was similar; the leaves droop, dry out, and turn brown, with darkening of the petiole in some cases. Browning of the vascular ring of affected stem bases and roots was seen in some cases, but was not always present. The disease as it occurred in the greenhouse was typically a root rot, as in almost all affected plants part or all of the crown or root tissue was decayed. As the season progressed more plants showed wilting, until by spring about half the plants were diseased in varying degrees. V. albo-atrum was found on dead portions of most of the diseased plants. The symptoms were complicated in the greenhouse by the activities of numerous sow-bugs, root maggots, and centipedes.

Inoculation experiments.

An experiment was set up to test the pathogenicity of V. albo-atrum

to potted mature clover plants under greenhouse conditions during the winter. Following the method of Bewley (1922), the soil was removed from part of the root system, and the tap root surface sterilized. A strip of tissue was raised with a flamed scalpel, some inoculum added from an agar culture, the tissue replaced, covered with moist cotton, and the soil packed down around the roots. Twenty-four plants were inoculated in this way, and 6 plants, to which no inoculum was added, were used as checks. At the end of 4 months, the check plants were still healthy. Of the 24 inoculated plants, 11 were dead, in 11 others the root area around the point of inoculation was rotted, and part of the top was dead, and 2 plants appeared quite healthy. V. albo-atrum was growing abundantly on the decayed portions of 12 of the plants. Periodic observations on the plants during this interval showed the development of the disease to be quite typical, as previously described.

To determine if V. albo-atrum could invade leaf tissues of red clover, the following experiments were conducted in the laboratory during the summer. Young clover plants in 2 inch pots were atomized with a heavy suspension of spores in water, then placed under bell jars for 10 days. Check plants were atomized with sterile water and placed under bell jars. The inoculated plants were dead at the end of the 10 day period, and V. albo-atrum grew freely on leaves and petioles. The check plants were fairly healthy at the end of the experiment. As only 4 plants were inoculated in this original experiment, it was repeated the summer following, using 10 check plants, and inoculating 30 plants. In 7 days, V. albo-atrum was

observed fruiting on some diseased leaves in the inoculated series, while all the checks were healthy. In 12 days, the inoculated plants showed a preponderance of dead leaves, a few healthy ones, and the rest with 1 or 2 leaflets dead or diseased. V. albo-atrum was growing on most of the dead tissues. Some of the leaves on the check plants were also dead at the end of the experiment, but they were few in number, and in several cases showed a heavy growth of mildew, to which death might have been due. In no case was V. albo-atrum found on check plants.

Kendrick and Schroeder (1934) used 2 methods of inoculating soil with Verticillium sp. causing a wilt of melons, both of which were adapted for use with clover. Duplicate tests were run, using soil which had been autoclaved at 20 pounds pressure for 2 hours, and unsterilized soil. V. albo-atrum on a mixed cereals medium was added to both lots of soil; sterile medium was added to one set of check pots, and another set received no treatment. Clover plants 3 to 4 months old were set into 6 inch pots of prepared soil, and were grown in the greenhouse during the winter. Within 4 weeks 2 plants in the inoculated series, 1 growing in sterilized and the other in unsterilized soil, developed typical wilt symptoms; all the rest were healthy. A wilted plant, and a healthy plant from the same series, are shown in Fig. 15. The inoculum was so abundant in both pots that the fungus can be seen fruiting on the surface of the soil and on grains of cereal. At the end of 5 weeks, 7 of the 16 plants inoculated were wilted, and 3 of 8 plants growing in

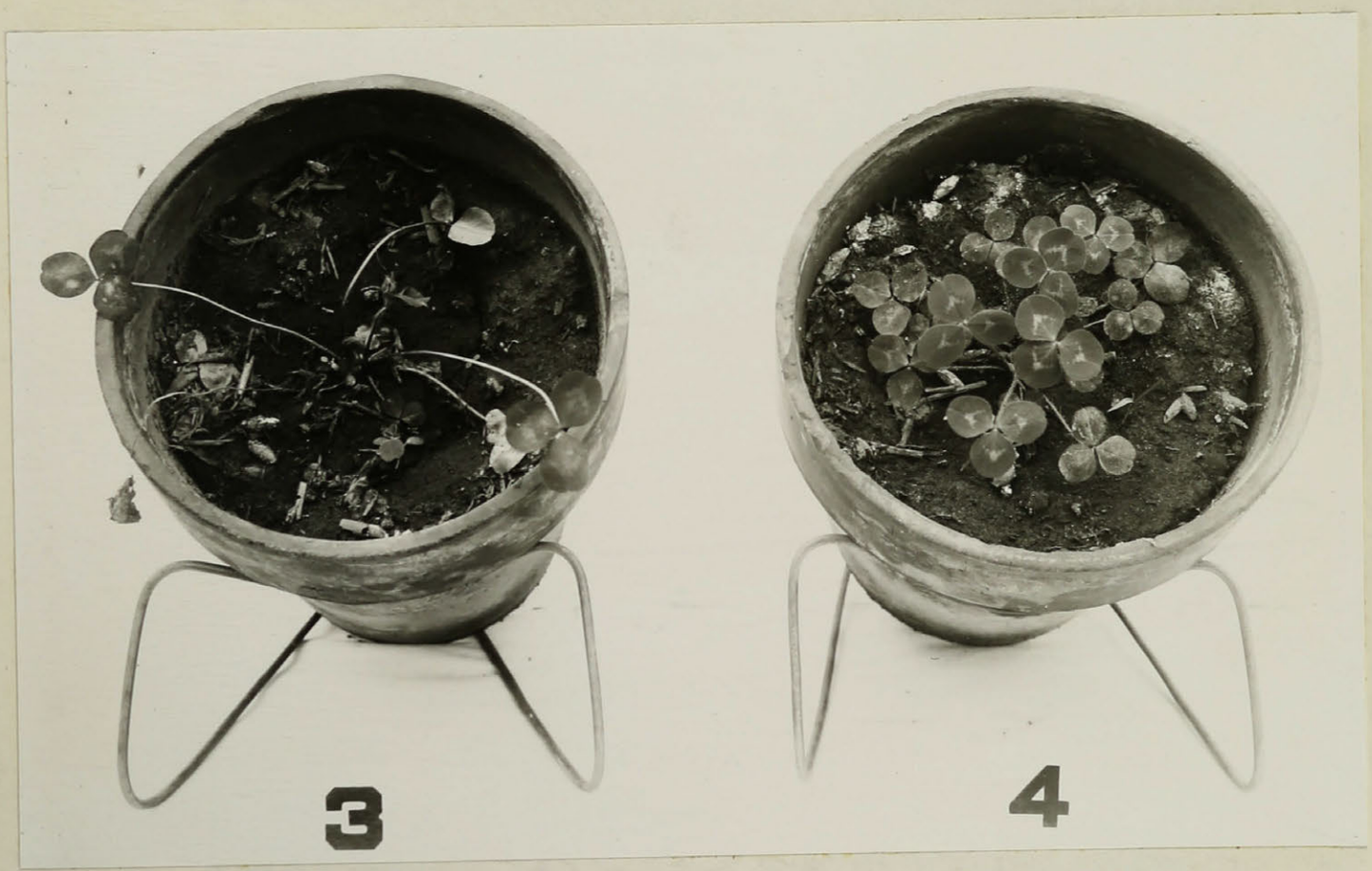

Fig. 15. Wilted plant and healthy plant from V. albo-atrum inoculation experiment.

unsterilized, uninoculated soil showed wilt symptoms. All 8 check plants in sterilized soil were healthy. The plants were taken up at the end of 7 weeks, and their roots examined. No root rot was present in any of the wilted plants; the root systems of 2 such plants appeared stunted and brown, but in most the diseased plants the roots appeared quite healthy. V. albo-atrum was not seen growing on any of the roots, confirming the impression that it fruits only on dead tissues of clover.

Inoculations made by the second method consisted of adding a heavy suspension of V. albo-atrum in water to soil in 4 inch pots. into which young plants of red clover had just been seeded. No conclusions could be drawn safely from the experiment, however, due to severe insect injuries on the leaves and roots of all the plants, making it impossible to distinguish symptoms of disease with certainty.

A rapid method to test pathogenicity of Fusarium spp. to tomato plants was published by Wellman in 1939. An adaptation of this method was used in inoculation experiments on red clover plants, with Verticillium albo-atrum, Fusarium avenaceum, and Sclerotinia trifoliorum. Soil mixed with 2 parts sand to 1 part soil was autoclaved at 20 pounds pressure for 2 hours, in 6 inch pots, then allowed to stand for several days. Inoculum was prepared by growing the fungi on a liquid medium described in Wellman's article, then preparing a water suspension of the mycelium by the use of an egg-beater. The soil was washed off the roots of the young clover plants,

they were soaked in the appropriate fungous suspension, and planted directly in prepared pots, which were then well watered. Check plants were washed and planted in the sterilized soil. Four plants were used in each pot; 36 pots were inoculated with V. albo-atrum; 15 with F. avenaceum; 15 with S. trifoliorum, and 15 were used as checks. One third of each series was kept in a cool, dark basement laboratory, one third in a greenhouse, and the remainder of each series was put outside. All pots were watered when the soil appeared dry. As the experiment was started in the summer time, the temperature was high throughout the experiment.

The table below shows the results of readings made 8 days after inoculating.

Table 13. Results of clover root inoculations by Wellman's method.

Location of plants	V. albo-atrum			F. avenaceum			S. trifoliorum		
	Dead	Wilted	Healthy	Dead	Wilted	Healthy	Dead	Wilted	Healthy
Laboratory	1	9	38	12	4	4	2	6	12
Greenhouse	3	4	41	8	5	7	0	5	15
Out doors	0	4	44	11	3	6	0	1	19

The check plants were all healthy. Additional readings were made at intervals for 6 weeks, but no appreciable change in the number of plants dead or wilted was apparent in the tests in the greenhouse and outdoors. The laboratory test had to be discarded after 2 weeks because of lack of light for the plants. The plants were all

taken up and examined 6 weeks after inoculation. Even though they appeared fairly healthy, it was observed that many of the inoculated plants were smaller and less vigorous than those in the check series. Cross sections of roots showed that the vascular systems of many of the plants inoculated with F. avenaceum showed a brown discoloration, although the plants were not visibly diseased. Several plants in the S. trifoliorum series showed similar discoloration of the vascular system, but most of the plants in this series appeared healthy. It was noted that there were usually several dead leaves attached to the plants inoculated with F. avenaceum and V. albo-atrum, particularly the latter. This was not the case with the check plants, or those inoculated with S. trifoliorum. In the series inoculated with V. albo-atrum, the fungus was found fruiting at the base of the petioles of many of the dead leaves.

DISCUSSION AND CONCLUSIONS.

Discussion of Survey Results.

Surveys on spaced plantings of red clover at Macdonald College have shown an interesting seasonal development of wilting. In 1 block of 1 year old plants, no wilt was found in a June survey. In August, the same block showed over 3% of the plants wilted. The number of plants reported dead increased from 0.3% to 10.6%, and the number missing from 40.1% to 48.4%. Part of this increase in the numbers of plants dead and missing might be attributed to the mechanical effects of cultivation. It is suspected, however, that most of these plants were killed by either wilt or root rot. Kendrick and Schroeder (1934) refer to the rapid wilting of musk-melons affected with Verticillium albo-atrum; Wolf and Cromwell (1919), describing the effect of Sclerotinia trifoliorum on clover, mention that the plants succumb quickly. Rapid wilting and death of clover plants in the spaced plantation is assumed to account for the large increase in dead and missing plants; if the symptoms are visible for only a short time before the plant dies, it would account also for the relatively low percentage of wilted plants observed at any one time.

No wilted plants were seen in surveys on farm fields of red clover in the western part of Quebec. A possible explanation for this is the tendency of plants in a sward to spread and cover an area vacated by the death of one of the plants. Thus the wilting

and death of isolated plants in a farm field might be obscured by the growth of neighboring plants to fill the empty space. In a spaced planting each plant can be observed separately, and the disease or death of individual plants is seen readily.

Correlation of Temperature Effects on Clover and Fungi Studied.

Poulter (1939), in a study of wound healing in red clover stems, found that suberization of injured surfaces did not take place in the late fall, winter, or early spring. Fields of red clover which went into the winter with numerous unprotected injuries showed from 50 to 95% disease the following summer. In fields which were uninjured after September 1, only 1 to 10% of the plants showed disease the following summer. Cormack (1933) studied the factors influencing formation of wound work, which Poulter demonstrated was important as a protection against disease. Cormack found that no wound cork is formed by sweet clover and alfalfa plants at 1°to 3°C; it starts forming at 8° to 9°C, and is formed rapidly at 16° to 18°C. Young plants produced wound cork better than overwintered ones; freezing lessened the ability to produce wound cork.

Cormack (1933) found that a complete layer of wound cork could be formed in 18 days at 9°C, or in 8 days at 18°C. Studies here reported, on the effects of temperature on 3 organisms found associated with wilted clover plants, show that all 3 made good growth at 18°C. This temperature was close to the optimum for Fusarium avenaceum and Sclerotinia trifoliorum, although rather low for best

growth of Verticillium albo-atrum. S. trifoliorum grew rapidly at 8°C and 4°C, although the other 2 fungi grew very slowly at 8°C. It is apparent that all 3 fungi can grow at temperatures too low for rapid formation of wound cork by alfalfa and sweet clover plants. As red clover has temperature relationships similar to alfalfa and sweet clover, it may be argued that the fungi studied may invade injured root or stem tissues of red clover in the spring, before the plant has been able to form a barrier of wound cork.

Weimer (1930) was able to cause root injuries on alfalfa plants by freezing them. The importance of this type of injury in relation to attack by root pathogens is stressed by Dickson (1939). He believes that root rots of clovers and related plants are so closely associated with winter injuries, that the failure of many investigators to demonstrate pathogenicity of root rot organisms in inoculation experiments conducted during the summer growing season, may be explained on this basis.

Conclusions on Significance of Fungi Studied.

Verticillium albo-atrum.

V. albo-atrum is known to cause wilting of many plants. Rudolph (1931) found it reported in the literature on over 120 species in 35 families and 18 orders of plants. Five leguminous hosts are reported in his compilation; 1 of these, Medicago hispida, was found to be susceptible in North America. Richter and Klinkowski (1938) reported a Verticillium wilt of alfalfa and sainfoin (Onobrychis sativa) in Germany. Boning (1936) mentions that V. dahliae,

found on horse-radish, may attack many other plants, including clovers. Canadian Plant Disease Survey annual reports from 1921 to 1938 contain references to Verticillium wilt of various crops. Only 1 leguminous host has been reported in Canada; V. albo-atrum was found on the roots of several wilted sweet-pea plants at Kentville, N. S., in 1937.

Experimental data here presented show that V. albo-atrum is associated with many wilted clover plants, that it can cause disease of mature plants and seedlings under certain conditions, and that it can produce in culture substances toxic to red clover. On the basis of these results and the known pathogenicity of this organism on other hosts, it is concluded that some of the wilt of red clover plants at Macdonald College is due to V. albo-atrum.

Fusarium avenaceum.

Cormack (1937) reported Fusarium avenaceum as the cause of a typical root rot, particularly severe after winter dormancy, on alfalfa and sweet clover. It also produced light to moderate winter and summer infection on roots of Trifolium spp. This organism has been isolated frequently from diseased roots of leguminous plants, and is a common root parasite of cereals.

F. avenaceum has been found associated with wilted clover plants at Macdonald College. Inoculation experiments have shown it to cause wilting and death of young clover plants under the conditions of the experiment. These results, supported by the results of

other workers, suggest that this fungus may be responsible in part for the wilt of red clover plants observed at the College.

Sclerotinia trifoliorum

Sclerotinia trifoliorum was stated by Pape (1931) to be the most important fungous parasite associated with winter injury to clover in Germany, where it has been known to destroy from 75% to 100% of a crop. This fungus has been reported on clover in many parts of Canada, with varying amounts of injury attributed to it.

Field observations at Macdonald College previous to the start of this study indicated that S. trifoliorum caused considerable damage to red clover. Experimental inoculations here reported showed it to be pathogenic to seedling clovers grown on agar, but failed to prove its pathogenicity to older plants. On the basis of numerous reports by other workers, however, it seems safe to conclude that in some seasons S. trifoliorum may be responsible for wilting of red clover plants in the college plots. No satisfactory explanation can yet be offered for the absence of sclerotia of this fungus in the fall of 1938 and in 1939.

SUMMARY

The symptoms of wilting red clover plants in spaced plantings at Macdonald College are described. Isolations from diseased material in various stages yielded a large number of fungi and bacteria, most of which were discarded because of their irregular occurrence.

Verticillium albo-atrum was found associated with dead roots of red clover. Sclerotinia trifoliorum and Fusarium avenaceum were also found associated with diseased clover.

The temperature optima for the 3 fungi in pure culture were 28°C, 20°C, and 20°C, respectively. S. trifoliorum was found to grow at 4°C. All 3 fungi grew over a wide pH range.

The fungi grown on Richard's medium produced substances which made filtrates of the medium toxic to red clover plants. Heating the filtrates did not affect their toxicity. Seedlings of red clover, grown aseptically on nutrient agar, were inoculated with the organisms studied. All 3 fungi were pathogenic.

V. albo-atrum was inoculated into the roots of mature red clover plants in the greenhouse. It proved pathogenic. Inoculations with all 3 fungi by a method described by Wellman showed F. avenaceum and V. albo-atrum to be more pathogenic than S. trifoliorum under the conditions of the experiment.

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. I. H. Crowell for his sympathetic advice and criticism throughout the course of these studies. He is indebted also to Prof. J. G. Coulson for advice and assistance in the photographic work; to Mr. J. N. Bird of the Agronomy department, for supplying all the clover plants for inoculation studies; and to Prof. P. H. H. Gray for his permission to use the facilities of the Bacteriology department.

LITERATURE CITED.

Anonymous.
1921 - 1938 Canadian Plant Disease Survey. Annual Reports 1 - 18.

Ayers, G. W. and Hurst, R. R.
1939 Verticillium wilt of potatoes in Prince Edward Island. Sci. Agr. 19 : 722 - 735.

Bennet, F. T.
1928 On two species of Fusarium, F. culmorum (W. G. Sm) Sacc. and F. avenaceum (Fr.) Sacc. as parasites of cereals. Ann. Appl. Biol. 15 : 213 - 244.

Bewley, W. F.
1922 "Sleepy disease" of the tomato. Ann. Appl. Biol. 9 : 116 - 134.

Boning, K.
1936 Untersuchungen uber Meerettichkrankheiten und deren Bekampfung. Angew. Bot. xviii, 6, pp. 482 - 494. In Rev. App. Myc. 16 : 360 - 361 (1937).

Carpenter, C. W.
1918 Wilt diseases of okra and the Verticillium wilt problem. Jour. Agr. Res. 12 : 529 - 546.

Chaudhuri, H.
1923 A study of the growth in culture of Verticillium albo-atrum, R. et Br. Ann. Bot. 37 : 517 - 539.

Chester, F. D.
1890 Rot of scarlet clover. Del. Coll. Agr. Exp. Sta. Rept. 3 : 85 - 88.

Cormack, M. W.
1933 On wound cork in roots of Medicago and Melilotus. Proc. World's Grain Exhib. and Conf. Canada Vol. 2 pp. 256 - 262.

Cormack, M. W.
1937 Fusarium spp. as root parasites of alfalfa and sweet clover in Alberta. Can. Jour. Res. Sec. C. 15: 493-510.

De Haan, J. I.
1937 Untersuchungen uber das Auftreten der Keimlings-Fusariose bei Gerste, Hafer, Mais und Reis. Phytopath. Z. X, 3, pp. 235 - 305. In Rev. App. Myc. 16 : 805 - 806 (1937).

Dickson, J. G.
1939. Outline of diseases of cereal and forage crop plants of the northern part of the United States. Burgess Publishing Co. - Minneapolis, 259 pp.

Fergus, E. N. & Valleau, W. D.
1926 A study of clover failure in Kentucky. Kentucky Agr. Exper. Sta. Bull. 269 : 139 - 210.

Guba, E. F.
1934 Control of Verticillium wilt of eggplant. Phytopath. 24 : 906 - 915.

Kendrick, J. B. and Schroeder, F. R.
1934 Inoculation tests with Verticillium wilt of muskmelons. Phytopath. 24 : 1250 - 1252.

Linford, M. B.
1931 Studies of pathogenesis and resistance in pea wilt caused by Fusarium orthoceros var. pisi. Phytopath. 21 : 797 - 826.

Ludbrook, W. V.
1933 Pathogenicity and environal studies on Verticillium hadromycosis. Phytopath. 23 : 117 - 154.

Mix, A. J. and Vaughn, D. L.
1924 The range of toleration of hydrogen-ion concentration exhibited by Fusarium tracheiphilum in culture. Phytopath. 14 : 63.

Pape, H.
1937 Beitrage zur Biologie und Bekampfung des Kleekrebses (Sclerotinia trifoliorum Erikss.) - Arb. biol. Anst. (Reichanst) Berl. xxii, 2, pp. 159 - 247. In Rev. App. Myc. 17: 252 - 253, (1938.)

Poulter, R. W.
1939 Problems of wound healing in red clover stems. Science 90 : (2327) : 108 - 109.

Rabinovitz - Sereni, D.
1932 L'azione dei raggi luminosi visibili di cazione e sulla pigmentazione dei funghi in coltura pura. Boll. R. Staz. Pat. Veg. N.S. xii 1, pp. 81 - 114. In Rev. App. Myc. 11 : 736 - 737 (1932).

Richter, H. & Klinkowski, M.
1938 Wirtelpilz - Welkekrankheit an Luzerne und Esparsette. Nachr Bl. dtsch. Pfl Sch Dienst. 18 : 57 - 8. (Biol. Reichsanst. Berlin-Dahlem).

Riker, A. J. and Riker, R. S.
1936 Introduction to research on plant diseases. John S. Swift, Co., 117 pp.

Rudolph, B. A.
1931 Verticillium hadromycosis. Hilgardia 5 : 197 - 361.

Sarejanni, J. A.
1938 La verticilliose du Coton en Grece - Ann. Inst. Phytopath. Benaki, Greece ii, 2 pp. 79 - 85 - 1936. In Rev. App. Myc. 18 : 26, (1939.)

Stan, G. H.
1932 A study of diseases of canning crops (peas and corn) in Minnesota. Minn. Agr. Ex. Sta. Tech. Bul. 89: 1-51.

Stevens, F. L. and J. G. Hall,
1911 A serious lettuce disease and a method of control. N. Car. Tech. Bul. 8 : 89 - 143.

Tu, C.
1930 Physiologic specialization in Fusarium spp. causing headblight of small grains. Univ. Minn. Agr. Exp. Sta. Tech. Bul. 74 : 27 pp.

Verona, O. and Ceccarelli, A.
1935 Su di una tracheomicosi dell Amaranto (Amaranthus tricolor L.) prodotta da una specie di Fusarium e da Verticillium amaranti n. sp. e, in genere, sulla biologia di alcuni Verticillium patogeni. Phytopath. Z., viii, 4, pp. 373 - 400. In Rev. App. Myc. 14 : 765 (1935.)

Weimer, J. L.
1930 Alfalfa root injuries resulting from freezing. Jour. Agr. Res. 40 : 121 - 143.

Wolf, F. A. and R. O. Cromwell,
1919 Clover stem rot. No. Caroline Ag. Ex. Sta. Tech. Bul. 16.

Young, W. J.
1923 Clover root rots and powdery mildew. Ohio Agr. Exp. Sta. Monthly Bul. 8 (10) : 157 - 160.