FUNGI ASSOCIATED WITH OREGON GROWN CLOVER SEED

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

FUNGI ASSOCIATED WITH OREGON GROWN CLOVER SEED

CHAPTER I

INTRODUCTION

The occurrence within the state of Oregon of regions ideally suited for seed production has led to the development of a seed industry producing approximately one-seventh of the total production of grass and legume seed in the United States (82, p. 1). Oregon seed is shipped to many parts of the United States as well as being exported to foregin countries. To maintain high quality standards, much of the seed is field inspected and laboratory tested for varietal purity, germination capacity and freedom from weed seed. However, seed is rarely examined, if at all, for phytopathogenic organisms even though it has long been recognized that important plant pathogens may be carried by the seed. The presence of plant pathogens with seed is important because it enables disease spread to new regions, and contributes to the survival of the pathogen from season to season. Historically, seed transmission of plant diseases has received inadequate attention by phytopathologists with the result that many diseases probably have attained world wide distribution through seed.

In Europe recently, there has been a movement toward the establishment of seed certification standards for freedom from disease (88). This movement is a step in the right direction for

without certification for freedom from disease, other phases of certification and seed testing fall short of their full purpose.

If the time should arrive when either public agencies or individual buyers, demand a certificate of seed health (17, p. 280), Oregon seed producers should be in a position to know what plant pathogens are carried by their seed. Limited standards already have been set up in Europe for certification of freedom from disease (88). If Oregon is to maintain its position in production and marketing of high quality seed, effort should be taken to assure that disease-free, certified seed is shipped from the state. To accomplish this we need to know much more about the organisms carried by seed of the different crops. This need for information on seed-borne organisms has motivated the current investigation.

The clovers (Trifolium spp.) comprise a major segment of the Oregon seed industry. The annual value of the clover seed crop alone approximates 9½ million dollars of an industry wide total of 26 million dollars (80, p. 3). With the economic value of the different clover species as the criterion of importance, it was decided to concentrate this work on the following major species: red clover (Trifolium pratense L.), alsike clover (T. hybridum L.), ladino clover (T. repens f. giganteum Lagr.-Foss.), and crimson clover (T. incarnatum L.) (39).

The primary objectives of the investigation were: to review under one cover, the main literature treating both pathogenic and non-pathogenic organisms associated with clover seed, to determine

experimentally the organisms associated with the seed, and to study the pathogenicity of suspected pathogens. Secondary objectives included determination of the positional relationship of fungi with the seed, the respiration of contaminated seed, the relationship between air-borne inoculum and seed contamination, the longevity of seed-borne organisms, and the effect of heat treatment on the seed microflora.

CHAPTER II

LITERATURE REVIEW

Though for a number of years Oregon has been one of the leading producers of clover seed, there has been little attention paid to the micro-organisms associated with this seed. No records were found which dealt specifically with fungi of Oregon grown seed, other than a report by Hardison[1] (38) on Botrytis anthophilla Bond. In other geographic regions however, considerable attention has been given clover seed and a numerous and widely scattered literature has accumulated.

For convenience, the articles reporting fungi associated with clover seed and those relating to seed treatment, are reviewed under the following headings: bibliographies, general surveys, information on specific micro-organisms, and heat treatment.

Bibliographies

The following bibliographies were found to be useful for tracing literature concerned with micro-organisms associated with clover seed:

The bibliography on seed by Franck and Bruijning includes a section devoted to seed-borne organisms of legumes (29, pp. 239-241) which covers all records up to 1931. Organisms reported since 1931,

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[1] Dr. J. R. Hardison in several unpublished reports (37), has noted the presence of sclerotia with Oregon grown clover seed.

are cited in a bibliography by Porter (85, pp. 303-310) accompanying a general review of seed-borne organisms. Orton, in his bibliography of "Seed-borne parasites" cites a number of references dealing with clover seed. A bulletin by Chilton et al. (11), though not directly concerned with fungi associated with seed, provides a means of tracing source material for the diseases of clover species. This bulletin contains records of all fungi reported on species of Trifolium up to 1943, and includes a bibliography of 1733 papers.

Though none of the bibliographies was complete in its coverage, together they served as a foundation from which the writer was able to build a knowledge of the micro-organisms associated with clover.

General Surveys

Most studies of micro-organisms associated with clover seed, have been limited to specific organisms. This is to be expected in as much as research usually has been on specific clover diseases, with seed transmission but one facet of the host-parasite relationship. However, a few workers have attempted to determine the overall picture of micro-organisms associated with clover seed.

Chilton (9, pp. 738 and 739) employing the agar plate method, examined surface disinfected red and subterranean clover seed for associated fungi. Fungi isolated from red clover seed less than a year old included; Pleospora herbarum (Pers.) Rabenhorst, Stemphylium sarcinaeforme (Cav.) Wiltshire, Cercospora zebrina Passerini, and the "black patch" fungus. A two per cent infestation of P. herbarum in one sample of red clover, was the highest recorded

incidence of any fungus. It was necessary to plate out 1,635 seeds of a sample to demonstrate the presence of the "black patch" fungus. Also present in the red clover were species of Phoma, Fusarium, Penicillium, Oospora and Chaetomium. No fungi were found in seed samples that were two or more years old. From subterranean clover, Sclerotinia bataticola Taub., Rhizoctonia solani Kuehn and Fusarium were isolated. Chilton probably obtained only an incomplete picture of the microflora of the above two clovers because of the severity of his disinfection procedure.

Evans (27) examined red clover seed from 14 countries to determine whether or not Gloeosporium trifolii Peck was present on the seed. Gloeosporium was not detected, but fungi of the following genera were observed: Penicillium, Stemphylium, Alternaria, Aspergillus, Botrytis, Mucor, Cladosporium, Fusarium, sterile mycelia and a sclerotium forming fungus. Evans observed that shrivelled and brown colored seeds showed a higher incidence of molds than did plump seeds of good color.

In a laboratory analysis of 24 samples of Trifolium pratense, T. repens, and T. hybridum, Rodionova (89, p. 115) reported that an average of 62.4 to 99 per cent of the seed samples were infested from 0.6 to 52 per cent by bacteria (unspecified), from 0 to 36.6 per cent by species of Fusarium, and from 25.5 to 98 per cent by molds, which were chiefly Mucor and Alternaria. Disinfection with potassium permanganate considerably reduced, but did not eliminate the fungi indicating that some of the organisms were carried internally.

Sigrianski and Otpushtshennikova (105) listed the organisms they found on red clover seed including Botrytis anthophilla, Fusarium sp., Cladosporium sp., and Penicillium sp. Doyer (25, p. 519) observed that clover seed at the Wageningen Seed Testing Station was commonly contaminated by Botrytis cinerea, B. trifolii, Fusarium sp. and Ascochyta imperfecta. Rice (86) reported the occurrence of Actinomucor and Trichurus on red clover seed.

Information on specific organisms

Many fungi are known to be associated with clover seed; most of these are species of little pathological significance yet all must be identified and considered when encountered. The object of the ensuing review is to gather information on specific microorganisms, so that it may be considered as a whole, rather than as so many isolated facts.

Acremoniella atra (Corda) Sacc.

Groves and Skolko (33) report isolation of Acremoniella atra, from seed of Trifolium hybridum. Included in this paper are descriptions of the fungus.

Alternaria tenuis auct. sensu Wiltshire

Alternaria tenuis is commonly found on seed of many plant species (75, p. 87) and has been reported on Trifolium hybridum and T. pratense (35, p. 219). Fergus and Valleau (28, p. 197) were able to isolate A. tenuis from surface sterilized clover seed.

There are reports of this fungus appearing as a weak parasite

on a great many plant species. Behrens (cited by Neergaard 75) carried out pathogenicity tests on T. pratense with negative results. According to Neergaard (75, p. 92), the fungus generally thrives as a saprophyte on dead and weakened plant parts.

Groves and Skolko (35) as well as Neergaard (75) provide information useful for the identification of A. tenuis.

Ascochyta imperfecta Peck (syn. Phoma medicagnis Malbr. and Roum.)

Ascochyta imperfecta is best known as the cause of "black stem" and is of economic importance in certain areas where losses of alfalfa (Medicago sativa) as high as 40-50 per cent are recorded (84, p. 591). The fungus attacks other species of Medicago as well as Melilotus and Trifolium species (13, p. 838). Sampson (92, p. 63) states that the host range is probably not fully known. Melchers (67, p. 183) reports A. imperfecta isolated from clover, and indicates that the fungus was the cause of a leaf spot of red clover (Trifolium pratense). Hardison (cited by Corden 12, p. 11) has on occasions isolated this fungus from rootlets of clover grown in Oregon.

Only one report was noted (25, p. 519) of A. imperfecta as a seed-borne pathogen of clover. (A. imperfecta was isolated on a number of occasions from seed of Trifolium species during the course of this investigation.) On alfalfa seed it was suggested by Cormack (13, p. 843) that A. imperfecta persists mainly as mycelia on or in the outer portion of the seed coat. He was unable to find pycnidia on infected pods or seed, nor was the fungus observed during routine examinations of several seed samples of sweet clover, red clover,

and alsike clover. A. imperfecta when carried on alfalfa seed was shown by Mead (65, p. 504) to cause a severe blighting of seedlings growing under moist conditions (up to 90 per cent blighted). He showed the fungus to be seed-borne in 75 per cent of the seed samples received from the alfalfa seed growing areas of Saskatchewan (65, p. 500). Cormack (13, p. 850) in longevity studies of A. imperfecta on alfalfa seed stored at room temperature, observed 24 per cent infestation after one year, 16 per cent after two years, and 6 per cent after 3 years. After 4 years the fungus could not be isolated.

Morphological and cultural descriptions, with accompanying measurements of spores and pycnidia are given by Peterson, Sampson and Toovey (84, p. 593; 92, p. 63; 104, pp. 707 and 709) and are useful for the identification of A. imperfecta.

Pseudomonas cerasi Griffin (syn. Bacterium trifoliorum Jones et al) (24, p. 90)

Pseudomonas cerasi causes a leaf spot of clovers, though it may also attack petioles, stipules and stems. Jones et al. (50, p. 50; 51, pp. 471-490) report that lesions from Bacterium trifoliorum have not been observed on the floral organs of clover, but that they are of common occurrence on the flower pedicels. It is believed that there is ample opportunity for seed to become contaminated either while yet in the field or during harvesting and threshing. That seed serves as a primary source of infection is indicated by the occurrence of diseased plants in newly planted pastures and lawns. Six species of clover are listed as hosts of B. trifoliorum.

Jones (51, pp. 475 and 476) gives information useful for the identification of this bacterium.

"Blackpatch"--a sterile fungus

"Blackpatch" is seed-borne (59, p. 16) and may cause a seedling blight. Fruiting structures of the "Black patch" fungus have not been found and consequently the taxonomic position of this fungus is not known. Krietlow (59, p. 16) reports that in warm, humid weather it attacks red and white clovers as well as other legumes. It does not cause widespread damage though occasional outbreaks have resulted in local losses. Leach and Elliott (62, pp. 1041 and 1047) state that the fungus caused severe injury to red clover and other legumes in West Virginia during 1949, and that it may reduce seed yield by 50 per cent. Their work is summed up with the statement, "...that blackpatch may, under favorable conditions, attack severely the stems, flowers, and seed of red clover and greatly reduce the yield of both hay and seed. The fungus is seed-transmitted and may cause a blight not only of those seedlings arising from the infected seeds, but also of seedlings from non-infected seeds that may be growing in close proximity to infected seeds."

Tenney (102, p. 68) observed mycelium clinging to the seed coat and reported that the mycelium may be found on the surface of seeds which otherwise appear healthy. In 1949 Leach and Elliott (62, pp. 1043-1047) examined seeds from heavily infected clover heads, as well as those showing various degrees of infection by the "blackpatch" fungus. Some seeds showed evidence of being infected while immature.

Frequently traces of coarse mycelium of the "blackpatch" fungus could be recognised immediately under the seed coat. Other seeds appeared to have been attacked at various stages of maturity. In some plump and apparently uninjured seed, coarse black hyphae radiated out over the seed from a central invasion point at the hilum. Seeds that were not appreciably shrivelled, but which had "blackpatch" mycelia on the seed coat, usually germinated normally, but the seedlings were soon killed by the fungus. It was noted that mycelia of species of Cladosporium and Alternaria on the seed coat, were very similar to that of "blackpatch". Seedlings from infected seeds were promptly killed, and if the humidity was high, the mycelium spread to adjacent seedlings and infected them. Seeds selected under the microscope as being free of visible mycelia were usually not infected and produced healthy seedlings. In studies on the pathological histology of "blackpatch" in relation to seed transmission in red clover, Tenney (102, pp. 68-71) observed that the fungus was present both on the surface of seed from infected fields, and within the embryonic tissues of such seed. In sections of these seeds, the mycelia was evident in the endosperm and between the cotyledons. He reports that as the infected seed germinates, the mycelium grows into the hypocotyl and usually causes the death of the seedling. Tenney believes that infected seed may be responsible for the transmission of the disease to new areas. Surface-borne mycelia was also found to attack the seedlings at germination. Sections showed that the fungus penetrated by mechanical pressure. A holdfast was formed on the surface

followed by one or more hyphae forcing their way through the epidermis.

In preliminary seed treatment trials for infested clover seed, Leach (62, p. 1048) reports some reduction of the "blackpatch" fungus.

Chaetomium spp.

Chaetomium cochliodes Pall. is recorded on seed of Trifolium pratense L., and C. globossum Kunze, on seed of T. hybridum L. (95, pp. 790 and 795). Seed samples heavily infested with Chaetomium may show a correspondingly low germination, though the pathological significance of these species remains obscure (96, p. 270).

The works by Skolko and Groves (95; 96) are particularly useful for identifying Chaetomium species encountered.

Cladosporium spp.

Cladosporium species are commonly carried by seed, and are usually saprophytic. Unspecified species have been recorded on clover seed (27; 105).

De Vries (18) has monographed the genus Cladosporium. This monograph is indispensible for identifications of the species of this genus.

Colletotrichum trifolii Bain. and Essary.

Colletotrichum trifolii ("Southern anthracnose") is considered by some to cause the most destructive disease of clover of the Southern States, where it can destroy stands completely (59, p. 10). Montieth (74, p. 14) suggests that clover anthracnose is carried

with seed from infested fields. He reports, however, that the stem just beneath the flower is very susceptable, and that any infection near the flower usually affects the stem beneath. The development of such infections quickly cuts off the water and food supply for the blossoms with the consequence that seeds are not produced. He therefore considers it unlikely that the disease is transmitted within the seed to the extent often assumed. Montieth holds that the fungus is carried with seed, primarily as fragments of diseased plant. Butler in New South Wales (7) also thinks that the fungus is carried over from season to season on diseased clover fragments mixed with seed as well as on plant debris in the soil. Sampson (93, p. 127) artificially inoculated clover seed with Colletotrichum trifolii. This resulted in 68 per cent of the seedlings becoming infected and proved the possibility of contaminated seed giving rise to diseased plants. However, according to Sampson, positive evidence for the transmission of the disease by naturally contaminated seed has not been obtained.

Tiffany (103) and Grove (32, p. 227, and 236-237) present descriptions of C. trifolii which are useful for the identification of this fungus.

Curvularia trifolii (Kauffm.) Boed. (syn. Brachysporium trifolii)

Curvularia trifolii is reported to have caused considerable wilting and premature dying of leaves of ladino clover in eastern United States (6).

Groves et al. (34, p. 101) report the isolation of C. trifolii

on a few occasions from seed, though not from clover seed.

Fusarium species

Fusarium species have been shown to cause root rots of clovers and have been isolated from roots by various workers (60, p. 16; 14, p. 7; 12, p. 78).

Fusarium poae, F. herbarum (F. avenaceum), F. acuminatum, F. equiseti, F. moniliforme and F. graminearum, have all been reported associated with clover seed (60, p. 14; 25, p. 519; 9, p. 739; 27, p.1; 30, p. 589). Gordon (30, p. 589) concludes that most Fusarium species which commonly occur in the soil or in other habitats in the localities where seed is produced, may be encountered in seed if the atmospheric conditions are relatively moist. He believes that species such as F. poae, F. equiseti and F. acuminatum are weak pathogens, and that their presence in seed and dissemination by this means is relatively unimportant. The extent to which they are seed-borne is probably more dependent on relatively high humidity during the crop period than on inherent pathogenicity. On the other hand, Gordon considers that the presence of F. oxysporum should not be overlooked for though the species may exist as a saprophyte, there are numerous pathogenic strains. Likewise because of their recognized pathogenesis, the presence on seed of F. avenaceum, F. moniliforme and F. graminearum is believed to merit attention.

Publications by Wollenweber (113), Snyder (99; 100) and Gordon (30; 31) provide sources for identification.

Gloeosporium trifolii Peck

Grove, (32, p. 227) under a description of Gloeosporium trifolii states that this fungus causes a disease of clover and is no doubt introduced with seed. Evans (27) examined seed from 14 countries to determine if Gloeosporium trifolii was present. He was unable to find the fungus in any of the samples. Sampson (92, p. 68) states that evidence suggests that G. trifolii Peck is a synonym of Stagonospora recedens (Massal.) Jones and Weimer.

Kabatiella caulivora (Kirchn.) Karak. (syn. Gloeosporium caulivorum Kirchn.)

Kabatiella caulivora ("Northern anthracnose" or "Scorch") is reported by Kreitlow, et al. (59, 9-10), to cause a major disease of red clover in the cooler parts of North America, Europe and Asia. Losses of 50 per cent or more are reported in some fields. Sampson (92, p. 66) states that a severe attack sometimes leads to a total failure of seed production.

There is still some controversy about the possibility of K. caulivora being seed-borne. Kirchner (cited by Sampson 93, p. 108) as early as 1902 suspected that the fungus might have been introduced into Germany on seed of French red clover. Minyaeva (71, p. 318) believes that K. caulivora can be seed transmitted by various means, and she observed that seed from infected plants bore spots. Baudys (5, p. 352) also holds that the fungus is carried by seed. Kreitlow et al. (59, p. 10) state that there is no conclusive evidence that K. caulivora is transmitted on or in the seed. Sampson (93, p. 124)

carried out tests on artificially inoculated seed which provided evidence of seed transmission of the anthracnose disease. However, she states that evidence based on naturally contaminated seed has not been obtained. The fact that shoots, infected just below the head, wither and produce no seed reduces the likelihood of seed transmission. Wellensiek (110, p. 99) grew 3000 plants from seed from 32 fields many of which were heavily infected with clover anthracnose. The results failed to support the view that anthracnose of clover is transmitted by seed. Unlike Sampson, Wellensiek found that artificially inoculated seed from healthy plants failed to become diseased. He considered it probable that fragments of diseased plant parts adhering to the seeds contribute to the spread of the infection.

Minyaeva (71, p. 318) showed that the viability of spores of K. caulivora on seed surfaces was significantly decreased by lengthening the storage period. After nine months, 0.28 per cent seed contamination was detected compared with 92.5 per cent at the beginning of the period. Sampson (93, p. 124) found that seed artificially infected with K. caulivora still had viable conidia after 18 months storage, though the viability of the conidia was reduced.

Sampson (93, pp. 121 and 123) and Grove (32, pp. 226-227), provide good descriptions of this fungus.

Ditylenchus dipsaci (Kuhn) Filipjev (syn. Tylenchus devestatrix, Kuhn)

Smith (98, p. 9) in a discussion of a nematode disease of red clover caused by Tylenchus devestatrix Kuhn, reports that nematodes

are carried to some extent in particles of clover plant which get into the seed in the threshing process. Seed which had passed through a cleaning mill, was found to be practically free from nematodes.

Nigrospora oryzae (Berk. and Br.) Petch.

Meir et al. (66, p. 415) observed N. oryzae on red clover seed. This fungus is commonly a weak, late season parasite on corn ears.

Mason (63) and Sprague (101, p. 406) give adequate descriptions for identification of N. oryzae.

Phoma trifolii Johnson and Valleau

Kreitlow et al. (59, p. 9) referring to Phoma trifolii, "spring blackstem", state that it may cause extensive damage during cool wet weather in the spring or fall.

No records were found of Phoma trifolii occurring on clover seed though seed transmission is suggested by Kreitlow (59, p. 9). As this fungus was observed on clover seed on a number of occasions during the present investigation, pertinent literature on P. trifolii is briefly reviewed below.

In Oregon, Hardison (cited by Corden 12, p. 10) suggests that Phoma trifolii is of little importance in alsike clover failure. Corden (12, p. 10) isolated the fungus from alsike clover roots, though it represented only 1 per cent of all Phoma isolates. When inoculated into alsike plants under greenhouse conditions it caused vascular discoloration and killed up to 40 per cent of the rootlets.

The original description and measurements by Johnson and

Valleau (46) are useful for identification of the fungus.

Pseudopeziza trifolii (Biv. - Bern.) Fuck.

Massee (64, pp. 65-67) reports that Pseudopeziza trifolii sometimes develops on the stems, sepals, and less frequently on the seed of clovers. She considers that in England the disease is mainly due to the use of badly cleaned and infected seed. In a sample of commercial seed the fungus was present in abundance on minute fragments of leaves, calyces, and rarely on the seed itself. Jones (48, p. 30, 31 and 34) discusses the possibility of seed transmission of both Pseudopiziza medicaginis and P. trifolii which is suggested by their wide distribution. In experiments with artificially inoculated seed, Jones reports that apothecia develop on the seed coat. He concludes, however, that there is no positive evidence of the method by which P. medicaginis gains access to remote alfalfa fields and there is limited evidence indicating that the fungus is not carried with the seed. Similarly, Sampson reports there is no clear evidence that this disease is seed transmitted (92, p. 69).

Pseudoplea trifolii (Rostr.) Petrak

The disease "pepper spot", caused by Pseudoplea trifolii according to Kreitlow (59, p. 11), is frequently observed on ladino and white clovers; though other clover species are attacked in the humid, temperate parts of the United States.

Hopkins (40, pp. 117, 118 and 125) considers infection of the calyx as significant in that it provides a means by which seed might

become infected and so transmit the pathogen. Seed infection would account for the general distribution of the suspected pathogen on a large number of commercial samples of white clover. However, Hopkins was unable to successfully isolate P. trifolii from the mycelium present in the seed coat. Kreitlow (59, p. 11) likewise holds that seed may become diseased when flower stalks and floral parts are attacked.

Though Hopkins was unable to definately establish the seed transmission of P. trifolii, Miles (69, p. 678) has positive evidence of the seed-borne nature of Pseudoplea medicaginis Miles on the seed of bur clover (Medicago hispida Gaertn.). Lesions on the seed of bur clover looked like small sclerotia, but possessed the structure of perithecia, although no asci were found in them. Miles considered that these sclerotia like structures on the seed were a means of transmitting the disease particularly as viable cultures were obtained from them.

Identification of Pseudoplea trifolii may be accomplished through reference to Sampson (92, p. 173), Jones (49, p. 299), Hopkins (40, pp. 117-126) and Horsfall (41, pp. 64-66).

Rhizoctonia solani Kuehn.

Chilton (9, p. 739) observed Rhizoctonia solani once in isolations from 380 seeds of subterranean clover (Trifolium subterraneum).

Sclerotial forms

There are a number of fungi associated with clover seed that

are capable of forming sclerotia--Sclerotinia trifoliorum, S. spermophilla, Botrytis cinerea, B. anthophilla, Mitrula sclerotiorum, and Typhula trifolii. Doyer (25, p. 519) summarizes the differences between sclerotia of S. trifoliorum, T. trifolii and M. sclerotiorum. She reports that at the Wageningen seed testing station clover seed had only once been contaminated by sclerotia of each of the first two named, while M. sclerotiorum had only been recorded in Denmark. Ekstrand (26, p. 298) notes the occurrence of both S. trifoliorum and M. sclerotiorum in clover seed. He observed fruiting bodies of Mitrula sclerotiorum emerge from sclerotia of S. trifoliorum and in his opinion M. sclerotiorum is not a parasite of clover, but of S. trifoliorum. Røed (90) corroborated Ekstrand's views for he observed a single sclerotium of the S. trifoliorum type, produce apothecia of both types and he states that M. sclerotiorum has invariably been found in association with S. trifoliorum. Evans (27) frequently found a sclerotial forming fungus in samples of red clover examined from fourteen countries. Nobel (78, p. 86) cites reports of Sclerotinia minor occurring on red clover, and S. sativa on alfalfa and sweet clover, though neither was reported as seed-borne. However, where there are sclerotia formed on or within plants, there is the possibility of transmission of the sclerotia mixed with seed.

Botrytis anthophilla Bond. (syn. B. trifolii van Beyma and B. antherarum trifolii Schlecht.)

Botrytis anthophilla ("Anther mold") has been reported by a number of workers as infecting clover seed. In 1923 van Beyma (108)

found the fungus very prevalent in red clover seed and named it B. trifolii. Subsequent investigators (78, p. 86; 94, p. 244) have concluded that B. trifolii is actually B. anthophilla. Silow (94, p. 245) isolated B. anthophilla from 3 months old red clover seed taken from infected plants. Juhans (52, p. 390) frequently observed this fungus on red clover seed imported into Esthonia from Poland, Lithuania and Livonia. World wide distribution of this fungus is given in a Commonwealth Mycological Institute map (19). The fungus is of economic importance because the pollen of infected plants is largely replaced by fungus spores with a consequent diminution of fertility (92, p. 84). Minyaeva (72, p. 115) reported that diseased plants of red clover are of the same height as healthy ones, but are lighter in weight and have fewer stems and heads. Hardison (38) found B. anthophilla on Oregon ladino clover from the Grants Pass area. He observed a single infected flower among several thousand examined. Hardison plated on potato dextrose agar 750 shrunken and shrivelled seed selected from the 1946 ladino clover seed crop and obtained less than one-half of one per cent recovery of this fungus. He states that "apparently the seed must be damaged before this systemic fungus can be isolated from infected seed on culture media." Silow (cited by Hardison (38)) indicated the occurrence of the fungus on red clover of American origin. The systemic infection of clover by B. anthophilla is stated by Bondarzew (cited by Silow 94, p. 242) to have its origin in an intraseminal mycelium, since seed though disinfected, gives rise to infected plants. He showed that

the intraseminal mycelium results from germination of conidia on the stigma at the time of pollination. He also suggests but did not prove, that bees transmit the fungus. Silow (94, p. 242) demonstrated experimentally that bees are often responsible for spore distribution. Sigrianski and Minyaeva (105, p. 440) report that single hyphae or clumps of mycelium of B. anthophilla may be easily detected in sections of infected red clover seed, mostly in the small cavity near the embryo. Preliminary germination experiments show that infection of the seed does not always lead to infection of the seedling. The fungus penetrates all parts of the plant but sporulates on anthers or sometimes in the gynoecium. The presence of the fungus in the ovary does not inhibit the production of seed, and the output of infected seed is therefore high. Bondarzew (cited by Silow 94, p. 245) actually traced the passage of the hyphae to the ovary cavity and infection of the young ovules. Primary infection of red clover is said to occur through the seed (66, p. 415). Secondary infection presumably is carried by insects especially bees, and possibly by wind. Bondarzew (cited by Silow 94, 245) reports conidia present in sediment from centrifuged seeds. Silow (94, p. 245) judging from knowledge of the life cycle of B. anthophilla feels that seedling infection is unlikely. In infected seeds the mycelium can best be detected in sections cut parallel to the cotyledons, where it is seen in the parenchymatous tissue under the seed coat especially near the future radical (72, p. 115). In the case of secondary infection in the field, the attack is confined to the flowers.

Troussova (106) reports that the incidence and severity of "anther mold" is considerably higher on the late flowering varieties of clover. This she attributes to the agency of bees which are most active in disseminating the fungus during the flowering stage. She also noted that the seed produced by diseased flowers is markedly smaller and lighter than from healthy flowers. Silow (94, p. 245) notes B. anthophilla developing extensively on three year old red clover seed which had lost its germinability. Sampson (92, p. 86) states that seed disinfection is unsuccessful as a control since the fungus is within the seed coat. As infected plants do not appear to be appreciably weakened, Sampson believes that from a purely agricultural point of view the need for preventive treatment does not arise. Meir (66) in contradiction to Sampson states that there is considerable reduction of primary infection of red clover plants by seed treatment.

Botrytis cinerea Pers.

Botrytis cinerea has been reported on clover seed on several occasions. Noble (78, p. 89) states that this fungus is apparently transmitted both in the form of mycelium in or on the seed, and as sclerotia which look remarkably like those of Sclerotinia trifoliorum. Flower infection is not unusual (47, p. 61; 115). Wormald (114, p. 28) records a few fructifications on the flower heads of red and white clover.

Sclerotinia spermophilla Noble

Alcock (1, pp. 31-36; 2, p. 13) in 1927 observed a fungus on

seed of Trifolium repens L. which he believes to be a "small" strain of Sclerotinia trifoliorum Eriks. Seeds are characterized by a peculiar grey pink color. Examination under low magnification (x16-20) revealed fungus mycelia occurring as shining flecks on the surface of the seed coat. Associated with this were brown slightly depressed areas on the affected seeds. Seed imported from New Zealand was infected by as much as 4 per cent by weight. The mycelia formed a loose mat under the seed coat. On oat agar mycelium grew out readily to give concentric rings of small sclerotia which were at first white and studded with drops of water. Sclerotia formed freely on seeds kept moist. Apothecia began to appear after approximately six weeks. Micro-conidia were also observed. Noble (78, pp. 84 and 90) described Alcock's fungus as a new species, Sclerotinia spermophilla. She states that S. spermophilla is not known to attack or inhabit any other part of the plant but seed, and even the means by which it reaches the seed is as yet unknown. A seed infected with this fungus fails to germinate and if kept damp, the resting mycelia will develop and give rise to a sclerotia. In this process, the rather flat looking seed swells and after a week or so the testa at one point bulges, and then breaks showing the developing sclerotium which projects from the seed. Noble (78, p. 87) found it difficult not to avoid the conclusion that B. anthophilla is the imperfect stage of a Sclerotinia, and considered it significant that while B. anthophilla had never been found on white clover, S. spermophilla had never been found on red. She states that S. spermophilla is unknown in the field,

although it occurs frequently on the seed. Seed infected with S. spermophilla fail to germinate whereas those infected with B. anthophilla do germinate.

Alcock (1; 2) and Noble (78, p. 84) provide descriptions of S. spermophilla.

Sclerotinia trifoliorum Erriks.

Sclerotinia trifoliorum causes one of the most serious diseases of forage legumes (59, p. 158-166; 92, p. 55), and in Britain mainly attacks broad red clover, though late flowering crimson, alsike and white clovers are sometimes affected. In Germany it was reported as the most important fungus parasite associated with winter injury of clover (83, p. 669). Sclerotinia "crown rot" has been the most widespread and damaging single disease of forage legumes in western Oregon (37, p. 23).

Sclerotia mixed with clover seed often have been observed in the seed laboratory (20; 21; 22; 23; 73, p. 60; 83, p. 669; 111; 112, p. 13). Fulton (112, p. 13) holds that seed contaminated with sclerotia would account for the introduction and presence of stem rot, and advised that seed samples should be sent to a seed testing laboratory to guard against new fields and localities becoming infested. Conflicting views are held about the importance of seed-borne mycelia and sclerotia in the spread of "clover rot" (92, p. 57). Troussova (cited by Sampson 92, p. 57) believed that the majority of the sclerotia found in seed samples belong to the fungus, Typhula

trifolii. She pointed out that the rotted plants usually do not produce seed and that resting bodies of S. trifoliorum in or close to the ground would not, therefore, be harvested. This is not in agreement with Pape's (83, p. 670) observations that sclerotia of S. trifoliorum are formed not only in root collar and stem base, but also on and in stems. In the course of mowing and threshing, the sclerotia from the stems become readily mixed with the seed, which they frequently resemble in size. Pape also holds that the fungus may be further disseminated by ascospores and mycelial fragments adhering to the seed itself, or occurring in minute portions of stems, leaves and pods contaminating the seed. He suggests that the larger sclerotia of the clover rot fungus would be easily removed from seed samples in the cleaning processes, but that it is certainly possible for smaller ones to remain in the finished seed and so carry infestation to healthy fields (92, p. 58). As early as 1878 Eriksson (cited by Wolf 112, p. 13) stated that S. trifoliorum undoubtedly over wintered as hyphae adhering to seeds and not as sclerotia or spores; and that the disease is spread by this means. Justham (53) was able to isolate S. trifoliorum only once from surface sterilized seed. However, Coleman (cited by Wolf 112, p. 13) states that sclerotia mixed with seed is the probable means of distribution of the disease, though distribution through spores adhering to the seeds is also a possibility. Noble (78, p. 89) is of the opinion that there is no evidence that S. trifoliorum is transmitted within seed as a mycelium, and that transmission is by means of sclerotia mixed with the seed.

For descriptions of S. trifoliorum, as well as discussions on taxonomic relationships, Keay (55), Purdy (87) and Noble (78, p. 84) are recommended.

Typhula trifolii Rostr.

Noble (79, p. 67) states that this fungus was first described by Rostrup in 1902, as a parasite of Trifolium pratense, T. repens and Medicago lupulin. The fungus forms small dark sclerotia as the resting stage. These sclerotia are so similar to Brassica seeds, that they often may be regarded as such when they occur mixed with clover seed. Rostrup (cited by Noble 79, p. 67) records sclerotia mixed with clover seed from East Prussia, Moravia, Hungary and Norway, and he considers the disease to be fairly common but unrecognized. Pape (83, p. 670) states that dark brown sclerotia of T. trifolii are often found among clover seed. On a number of occasions Dorph-Peterson (20; 21; 22; 23) reports the occurrence of sclerotia of this fungus mixed with clover seeds and both Jorstad and Doyer note T. trifolii in seed (cited by Noble, 79, p. 67).

Morphology and cytology of Typhula trifolii are discussed by Noble (79).

Sordaria fimicola (Rob.) Ces. and de Not.

Cain and Groves (8, p. 488) report that Sordaria fimicola was isolated from seed of 22 species of plants including Trifolium hybridum.

Stemphylium botryosum Wallroth (Perfect stage = Pleospora herbarum (Pers.) Rabenh.)

Stemphylium botryosum attacks a number of hosts both within and without the Leguminosae. On red clover it attacks mainly the leaf tissue, but it may also occur on stems and petioles (92, pp. 76 and 77). The fungus is a ubiquitous facultative parasite which is often found on dead and weakened plants (75, pp. 361-379).

Pleospora herbarum has been isolated by Groves and Skolko (36, p. 193) from a wide range of seeds including red clover. Smith (97, p. 835) states that S. botryosum infects seed of red, white, and alsike clovers. Chilton (cited by Sampson 92, p. 77) isolated both S. botryosum and S. sarcinaeforme from blemished seeds of red clover which had been surface sterilized. The fungus has also been reported on clover seed from Esthonia (52, p. 390).

The following authors provide information for identification of this fungus: Sampson (92, pp. 76 and 77), Neergaard (75, pp. 368 and 372), Smith (97, p. 836-838), Groves and Skolko (36, pp. 193-196) and Nelson (76).

Stemphylium sarcinaeforme (Cav.) Wilt.

Stemphylium sarcinaeforme is reported to cause slight damage to red clover in Britain though severe outbreaks have been recorded in the U.S.A. (92, p. 75). Both stem and leaves of red clover are attacked.

S. sarcinaeforme is seed-borne and can also survive on old plant parts or in the soil (59, p. 7). Groves and Skolko recorded the

fungus on red clover seed (36, p. 195). Milburn and Bessey (68, p. 72) reported that S. sarcinaeforme occurred inside the seed causing germination failure. Infected seed was somewhat shrunken, wrinkled, and much darker in color than healthy seed. Krakover (57, p. 285) is of the opinion that the fungus mycelium within the seed coat is not especially important in causing infection of young plants. He found that centrifuged seed revealed the presence of spores of S. sarcinaeforme. Krakover holds that the spore bearing seeds are merely a means of transferring the spores to the soil from which infection may take place by splashing.

Groves and Skolko (36, p. 195), Sampson (92, p. 75) and Neergaard (75, pp. 372, 381, and 382) provide identification material for S. sarcinaeforme.

Sterile mycelium

Evans (27, p. 1) recorded the presence of a sterile mycelium in red clover seed. Fergus and Valleau (28, p. 558) isolated a fungus from surface sterilized clover seed which produced a growth of rust-colored hyphae (See Epicoccum under Appendix A).

Verticillium species

No references were found which noted Verticillium occurring on clover seed, and there are only a few references to the presence of Verticillium species on the seed of any plant. As Verticillium was encountered in this investigation (Chapter III and Appendix A), a brief review is made of pertinent literature concerning fungi of

this genus on seed of other hosts.

Kadow (54, p. 1265) states that as far as he is aware there were only two references to seed as a possible carrier of Verticillium wilt. He cites Wollenweber as having isolated a Verticillium species from wheat seed in Germany, though the pathogenicity of this isolate was not established. Richardson (cited by Kadow) isolated a wilt-producing Verticillium species from eggplant seed. Kadow (54, p. 1268) reports that the Verticillium wilt fungus can be carried by tomato and eggplant seeds and that internal seed infection seems to be most important.

Heat treatment

The only record of heat treatment of clover seed observed by the writer, was that by Komarova (56) who in a study of clover anthracnose (Kabatiella caulivora), reported that the best results of disinfection tests were obtained with dry heat for six hours at 70°C, or four days at 60°C. These treatments are said to increase germination and reduce infection to innocuous proportions.

In view of the heat treatment trials performed during the current investigation, the following literature is pertinent. Miller (70, p. 87) in a consideration of the disadvantages of water as a medium for heat treating seed, tested a number of liquids as substitutes and found carbon tetrachloride to be the most suitable. The germination of peas and beans heated in carbon tetrachloride at 75°C for 60 minutes was not significantly affected. Cruickshank (16) was able to control two fungus pathogens of flax by steeping

the seed in carbon tetrachloride for various lengths of time at temperatures from 60 to 75°C. Crocker and Barton (15, p. 236) state that dry seed will withstand the temperature of boiling water for hours provided that it is heated in a vacuum or in an inert atmosphere such as nitrogen. This prevents oxidation of fats and proteins within the seed.

CHAPTER III

SURVEY OF MICRO-ORGANISMS ASSOCIATED WITH OREGON GROWN CLOVER SEED

The survey reported in this study was restricted to the four clover species most important in the Oregon seed industry: crimson clover (Trifolium incarnatum L.), red clover (T. pratense L.), alsike clover (T. hybridum L.) and ladino clover (T. repens f. giganteum Lagr.-Foss).

The initial plan was to report only phytopathogenic fungi. However, it was soon realized that this was not feasible. All fungi first had to be identified at least to genus before it was possible to categorize them tentatively as saprophytes or parasites. Those tentatively identified as pathogens or potential pathogens were held for pathogenicity tests.

Survey methods

A total of 78 samples of clover seed was examined. Of these 19 were red clover, 19 crimson clover, 20 alsike clover and 20 ladino clover. All seed was obtained through the state seed testing laboratory at Corvallis, Oregon; with the exception of 6 samples of red clover obtained from E. F. Burlingham and Sons of Forest Grove, Oregon. The seed lots examined had been sent to the laboratory by growers and seed processors for purity and germination tests.

Samples to be surveyed, were periodically selected at random from the seed laboratory's filed records of purity and germination

tests. The first selections were made through the autumn and winter of 1953, from records of crimson and red clovers harvested during the preceding season. However, most of the red clover samples, and all of the samples of alsike and ladino, were selected from purity and germination test records for seed harvested in 1954. Samples of seed corresponding to the filed records, were obtained from the seed laboratory's storage room after each selection. As the individual samples had been mixed for uniformity prior to purity and germination tests, no additional mixing was required. Five cubic centimeters of seed were withdrawn from each selected sample and stored in corked vials at room temperature until they could be examined. Samples selected in this manner, were representative of the clover growing regions of the state (Figure 1). Most samples originated from the Willamette Valley and Madras-Redmond areas, but some were from Elgin, Nyssa, Medford (Central Point), and Klamath Falls.

Two cubic centimeters of seed were removed from the 5 cc. sample and divided in half. One-half was surface disinfected for 8 minutes with a freshly prepared[2] mixture of sodium hypochlorite and ethanol. The other half was left untreated. Two hundred seeds from each of the halves were plated 13 seeds to a plate on 2 per cent malt extract agar. The plates were incubated at 21°C and examined seven to fourteen days after plating, when records were made of all fungi present.

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[2] 4 parts sodium hypochlorite (5.25 per cent), 1 part 94 per cent ethanol, and 8 parts of distilled water.

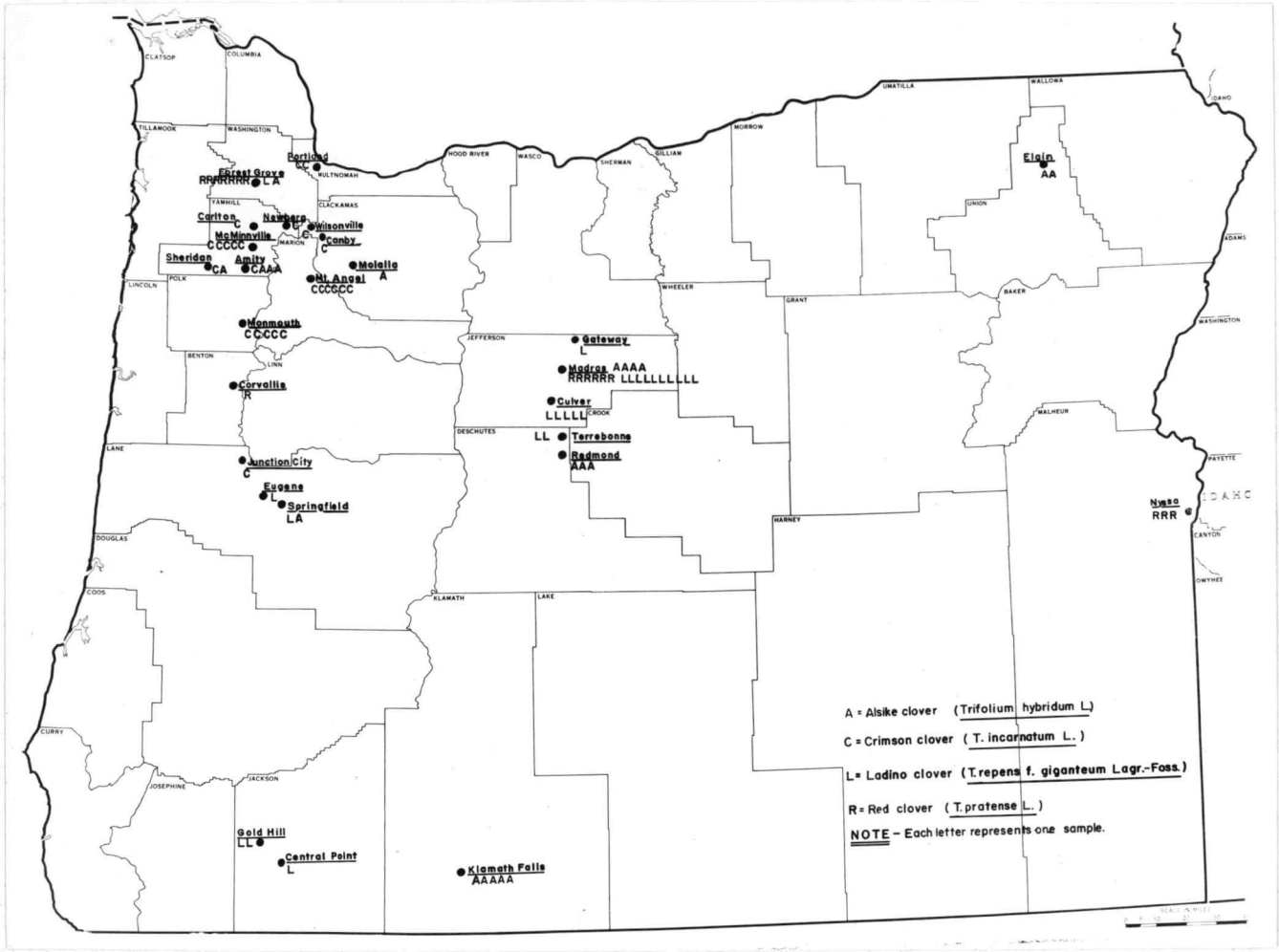

Figure 1. Source of clover seed samples examined for seed-borne fungi (seed harvested 1953 and 1954.

Most of the fungi fruited and could be identified after seven days. Colonies that were still sterile after the seventh day, usually produced fructifications by the fourteenth day.

Initial determinations were difficult because often there were half a dozen or more fungus species growing from seeds of a single plate, moreover the young colonies of a number of species were indistinguishable macroscopically. After some experience with the cultural characteristics of the different species, most of the fungi were identified from macroscopic characteristics. There were, however, numerous occasions when the macroscopic characteristics were not those of known fungi and it became necessary to examine cultures microscopically.

All species observed for the first time whether identified or not, were isolated, transferred to malt agar slants and then stored at room temperature in sterilized soil (3).

Equipment was devised to mechanize the disinfection and plating of seeds (61, pp. 94-96). The equipment included a seed disinfecting apparatus (Figure 2, 1A-C), an aseptic seed drier (Figures 2, 2A-2B) and vacuum counters (Figure 2, 3A-3B). Seeds to be disinfected were placed in wire mesh containers (Figure 2, 1C). The containers were inserted in the disinfecting apparatus and the seeds surface disinfected followed by three washes of sterile water. The seeds were then dried aseptically in a stream of filtered air, in the drier. Dry surface disinfected seeds as well as untreated seeds were plated on malt agar (Figure 2, 4) with specially designed vacuum counters

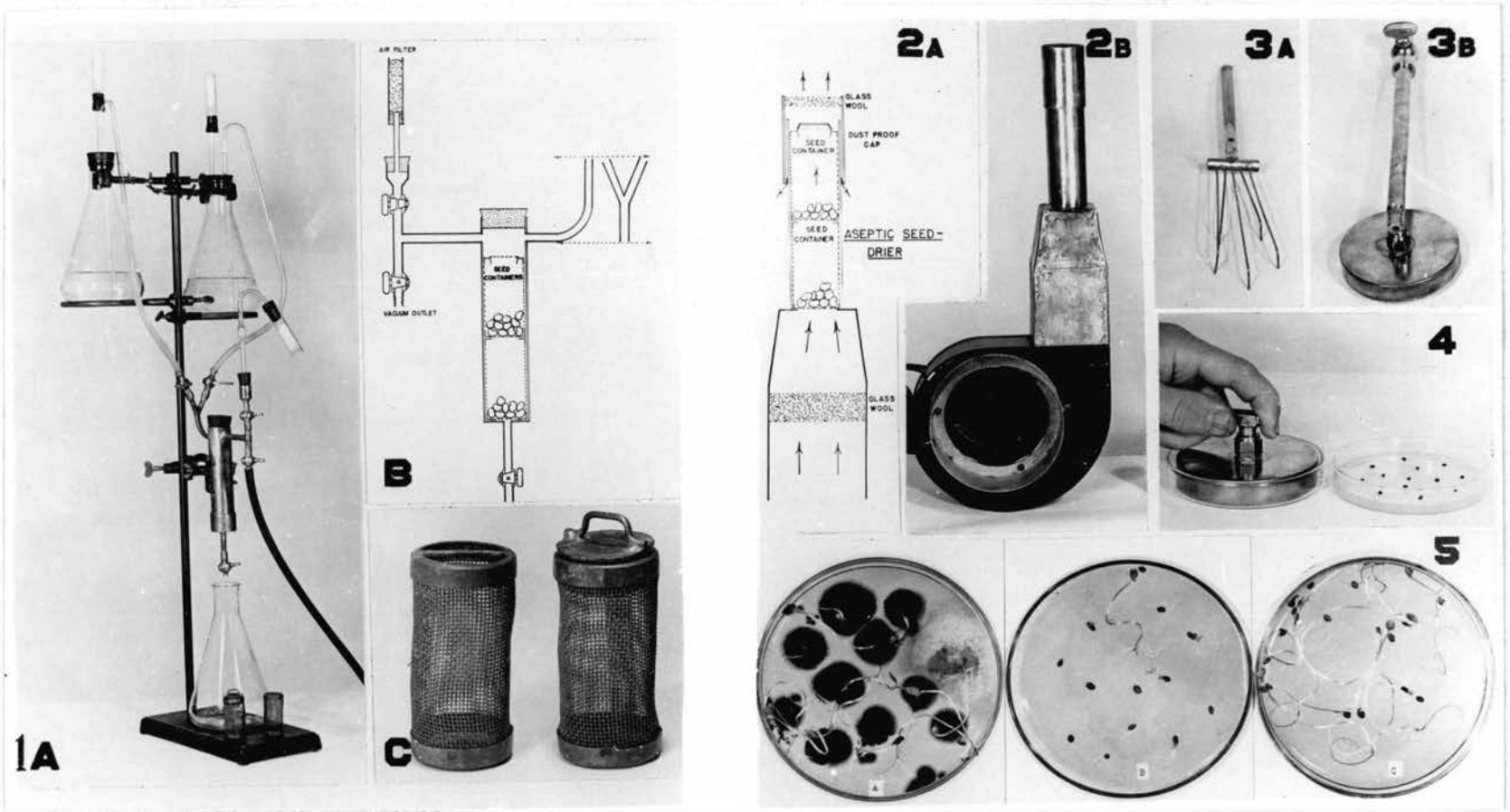

Figure 2. 1. Seed disinfecting apparatus. A)Complete apparatus. B)Sectional drawing through disinfecting chamber. C)Seed containers. 2. Aseptic seed drier. A)Sectional drawing through drier. B) Complete apparatus. 3. Vacuum seed counters. A)Claw type. B)Plate type. 4. Vacuum counter in use. 5. Clover seed 7 days after plating. A)Non-disinfected. B)Surface disinfected. (Photographs by H. H. Milsap).

(Figure 2, 3A-3B). The likelihood of introducing contamination while plating with counters, was lessened by employing a small glass sided transfer chamber (2½ ft. x 2½ ft. x 2½ ft.) sprayed with propylene glycol prior to use.

"Difco" 2 per cent malt extract agar made with tap water and having an unadjusted pH of 4.8-5.5 was used throughout. No cultural differences were discerned between media made with tap water on the one hand, and distilled water on the other.

Survey results

A total of 31,200 surface disinfected and untreated clover seeds was examined during this survey. Approximately 75 per cent of 15,600 untreated seeds were infested with micro-organisms, compared with only 15 per cent infestation of an equal number of surface disinfected seeds (Table II).

The following fungi were recorded:[3]

Alternaria tenuis auct. sensu Wiltshire
Ascochyta imperfecta Peck
Aspergillus fumigatus Fresenius
A. niger van Tiegham
Aspergillus sp. (identified as a member of the *A. ochraceus* group of thom and Raper)
Botrytis cinerea Persoon
Cephalosporium sp.
Cephalothecium sp.
Chaetomium cochliodes Palliser
C. funicola Cooke
Cladosporium cladosporioides (Fres.) de Vries

(continued on page 38)

[3]Many of these fungi are illustrated in Appendix C.

C. elatum (Harz) Nannfeldt
C. macrocarpum Preuss
Didymium difforme (Pers.) Gray
Epicoccum sp. (See Appendix A)
Fusarium roseum (Lk.) Snyder and Hansen (See Appendix A)
[4]F. oxysporum (Schl.) Snyder and Hansen
Mucoraceous spp.
Nigrospora oryzae (Berk. and Br.) Petch
Penicillium sp. (identified to a member of the P. javanicum series of Raper and Thom)
P. novae-zeelandiae van Beyma
Penicillium spp.
Phoma trifolii Johnson and Valleau
Pullularia sp.
Rhizopus sp.
Sclerotinia sp. (See Appendix A)
Scopulariopsis brevicaulis (Sacc.) Bainier
Stemphylium consortiale (Thum.) Groves
[4]Trichoderma sp.
Verticillium sp. (See Appendix A)
A yellow bacterium
A white bacterium (Bacterium trifoliorum Jones?)

Of the 78 samples examined, many were infested with potentially pathogenic fungi (Table I). The percentage occurrence of each of these fungi in the 78 samples was: Botrytis cinerea 47 per cent, Fusarium roseum 42 per cent, Stemphylium botryosum 36 per cent, Phoma trifolii 35 per cent, Ascochyta imperfecta 21 per cent, Sclerotinia sp. 10 per cent, Verticillium sp. 9 per cent, and Stemphylium sp. (Pseudoplea?) 6 per cent. Saprophytes were considerably more numerous than the pathogens, and were found in all samples. Ninety-seven per cent of the samples were infested with Alternaria tenuis and

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[4] Observed growing from plated, untreated clover seed after the survey had been completed.

ninety-six per cent with Cladosporium cladosporioides. On the other hand, a few of the saprophytic species, e.g. Chaetomium cochliodes, were found in only one sample.

In considering the overall infestation of individual clover seeds (Table II), the presence of Cladosporium cladosporioides greatly outnumbers all other fungi. It was present on nearly 60 per cent of the 15,600 plated untreated seeds and 11.5 per cent of the surface disinfected seeds. Alternaria tenuis was the next most numerous occuring on 9 per cent of the untreated seeds, followed by Cladosporium elatum on 5 per cent of the untreated seeds. Several organisms were found on only one seed out of the 31,200 examined.

As far as could be deduced from these results, the climatic and geographic influences of the various clover growing areas of Oregon (Figure 1), did not significantly influence the qualitative composition of the seed microflora. Nor were there appreciable differences between the micro-floras of red, crimson, ladino, and alsike clovers. There were some exceptions though, the Sclerotinia sp. was found only on crimson clover in the Willamette Valley (Hillsborough), and a species of Stemphylium (Pseudoplea?), only on white clover in Central Oregon. Seed samples carrying other potential pathogens were fairly representative of the clover growing regions of the state (Table III).

Table I. Frequency of clover seed samples infested by micro-organisms (Combined data from 1953 and 1954 seed crops).

Fungus	Number of infested clover samples				Percentage of all samples infested
	T. pratense	T. incarnatum	T. hybridum	T. repens	
	Number	Number	Number	Number	Per Cent
Alternaria tenuis	19[1]	19	18	20	97
Cladosporium cladosporiodes C. macrocarpum[2]	19	19	18	19	96
Penicillium spp.	11	11	17	13	67
Cladosporium elatum	10	-	14	18	54
Stemphylium consortiale S. sarcinaeforme[3]	13	12	7	10	54
Sterile mycelia	14	10	10	8	54
Mucoraceous spp.	8	9	10	12	50
Botrytis cinerea	11	14	8	4	47
Fusarium roseum	10	11	4	7	42
Epicoccum sp.	8	-	16	4	36
Stemphylium botryosum	7	10	4	7	36
Phoma trifolii	4	15	4	4	35

(continued on Page 41)

Table I. (continued) Frequency of clover seed samples infested by micro-organisms (Combined data from 1953 and 1954 seed crops).

Fungus	Number of infested clover samples				Percentage of all samples infested
	T. pratense	T. incarnatum	T. hybridum	T. repens	
	Number	Number	Number	Number	Per Cent
Bacterium (yellow	6	-	10	11	35
Aspergillus spp.	6	2	8	10	33
Bacterium (white)	1	-	10	8	24
Ascochyta imperfecta	-	9	5	2	21
Sclerotinia sp.	-	8	-	-	10
Pullularia sp.	2	3	3	-	10
Verticillium sp.	4	-	1	2	9
Stemphylium sp. (Pseudoplea?)	-	-	-	5	6
Scopulariopsis brevicaulis	-	-	-	3	4
Chaetomium cochliodes	-	1	-	-	1
C. funicola	-	-	-	1	1

(continued on Page 42)

Table I. (continued) Frequency of clover seed samples infested by micro-organisms (Combined data from 1953 and 1954 seed crops).

Fungus	Number of infested clover samples				Percentage of all samples infested
	T. pratense	T. incarnatum	T. hybridum	T. repens	
	Number	Number	Number	Number	Per Cent
Didymium difforme (Myxomycete)	1	-	-	-	1
Nigrospora oryzae	-	1	-	-	1
Cephalothecium sp.	-	1	-	-	1
Cephalosporium sp.	-	1	-	-	1

[1] 19 samples each of T. pratense and T. incarnatum, and 20 samples each of T. hybridum and T. repens were examined by plating on malt agar.

[2] C. cladosporioides and C. macrocarpum are quite similar culturally and for ease of counting were included together. C. cladosporioides was, however, far more numerous than C. macrocarpum.

[3] As in note 2, S. sarcinaeforme and S. consortiale were counted as one. S. consortiale was by far the more numerous of the two.

Table II. Frequency of micro-organisms associated with surface disinfected and untreated Oregon grown clover seeds (Combined data from 1953 and 1954 seed crops).

Micro-organism	Trifolium species examined								Total	
	T. pratense		T. incarnatum		T. hybridum		T. repens			
	Seed treated	Not treated	Seed treated	Not treated	Seed treated	Not treated	Seed treated	Not treated	Seed treated	Not treated
	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number
Alternaria tenuis	39[1]	207[1]	201	848	9	202	68	271	317	1528
Ascochyta imperfecta	-	-	7	34	-	6	-	3	7	43
Aspergillus spp.	13	10	-	5	1	20	2	34	16	69
Botrytis cinerea	-	26	22	41	8	41	1	3	31	111
Cephalosporium sp.	-	-	-	1	-	-	-	-	-	1
Cephalothecium sp.	-	-	-	2	-	-	-	-	-	2
Chaetomium cochliodes	-	-	-	1	-	-	-	-	-	1
C. funicola	-	-	-	-	-	-	-	1	-	1
Cladosporium cladosporioides (and C. macrocarpum)[2]	669	2836	663	1870	42	1683	195	1713	1569	8102
C. elatum	14	254	-	-	14	261	18	179	46	694

(continued on Page 44)

Table II. (continued) Frequency of micro-organisms associated with surface disinfected and untreated Oregon grown clover seeds (Combined data from 1953 and 1954 seed crops).

Micro-organism	Trifolium species examined								Total	
	T. pratense		T. incarnatum		T. hybridum		T. repens			
	Seed treated	Not treated	Seed treated	Not treated	Seed treated	Not treated	Seed treated	Not treated	Seed treated	Not treated
	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number
Didymium difforme (myxomycete)	-	1	-	-	-	-	-	-	-	1
Epicoccum sp.	11	8	19	188	-	10	-	4	30	210
Fusarium roseum	4	15	17	29	-	6	5	12	33	62
Mucoraceous spp.	3	27	1	23	2	17	2	16	7	83
Nigrospora oryzae	1	-	-	-	-	-	-	-	1	-
Penicillium spp.	24	146	2	41	7	56	7	27	40	272
Phoma trifolii	-	6	15	44	-	9	1	7	16	31
Pullularia sp.	1	2	1	2	2	4	-	-	4	8
Sclerotinia sp.	-	-	4	58	-	-	-	-	4	58
Scopulariopsis brevicaulis	-	-	-	-	-	-	-	13	-	13

(continued on Page 45)

Table II. (continued) Frequency of micro-organisms associated with surface disinfected and untreated Oregon grown clover seeds (Combined data from 1953 and 1954 seed crops).

Micro-organism	Trifolium species examined								Total	
	T. pratense		T. incarnatum		T. hybridum		T. repens			
	Seed treated	Not treated	Seed treated	Not treated	Seed treated	Not treated	Seed treated	Not treated	Seed treated	Not treated
	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number
Stemphylium consortiale (and S. sarcinaeforme)[3]	-	-	6	47	-	18	3	20	9	85
S. botryosum	-	-	38	134	-	5	7	3	45	192
Stemphylium sp. (Pseudoplea?)	-	-	-	-	-	-	7	21	7	21
Verticillium sp.	6	6	-	-	-	1	-	4	6	11
White bacterium	-	1	-	-	4	58	8	102	12	158
Yellow bacterium	5	30	-	-	12	35	16	28	33	93
									2,261	10,914

[1] Values are based on 19 samples each of T. pratense and T. incarnatum, and 20 samples each for T. hybridum and T. repens. Each sample consisted of 400 seeds--200 were plated on malt agar after surface disinfection, the other 200 were plated untreated.

[2] C. cladosporioides and C. macrocarpum are similar culturally and for ease of counting were included together. C. cladosporioides was however, far more numerous than C. macrocarpum.

[3] As in note 2, S. sarcinaeforme and S. consortiale were counted as one. S. consortiale was by far the more numerous of the two.

Table III. Geographic distribution of clover seed samples infested with potentially pathogenic fungi. (Combined data from 1953 and 1954 seed crops).

Fungus	Number of samples infested					
	Willamette Valley	Central Oregon	Klamath Falls area	Medford area	Nyssa area	Elgin area
	Number	Number	Number	Number	Number	Number
Ascochyta imperfecta	10	5	-	1	-	-
Botrytis cinerea	20	12	3	1	1	-
Fusarium roseum	17	10	-	1	2	1
F. oxysporum	1	-	-	-	-	-
Phoma trifolii	20	4	2	-	-	1
Sclerotinia sp.	8	-	-	-	-	-
Stemphylium botryosum	19	13	1	2	1	-
S. sarcinaeforme[1]						
Stemphylium sp. (Pseudoplea?)	-	5	-	-	-	-
Verticillium sp.	-	3	1	-	3	-
No. of samples examined from different locations.	34	31	5	3	3	2

[1] Because of cultural similarities between Stemphylium sarcinaeforme and S. consortiale, the two fungi were counted as one during the survey, and consequently no distribution of S. sarcinaeforme is given.

CHAPTER IV

PATHOGENICITY TESTS WITH SUSPECTED PATHOGENS ISOLATED FROM CLOVER SEED

The object of the ensuing pathogenicity tests was to evaluate the relative importance of the fungi isolated from clover seed. As diseases of cultivated clovers have been studied for many years, it is probable that the majority of important clover pathogens are now known. In view of this knowledge, it seemed unlikely that the preceding survey would reveal new parasites. Pathogenicity studies therefore were mainly confined to known clover pathogens.

Fungi isolated from clover seed during the survey, were initially screened for pathogenicity by inoculating them onto aseptic clover seedlings grown in test tubes. The more virulent forms as selected by these screening tests, were inoculated in the greenhouse and field, and a few fungi were tested as foliar pathogens.

Materials and methods

During the survey, suspected pathogens were not isolated on each occasion of their occurrence on plated seed. Instead representative fungi were selected, thus reducing the labor of carrying large numbers of cultures. Fungi were selected as follows: where a specific fungus was observed growing from more than one seed of a plated sample, isolations were made from only one, or at the most two of the colonies. If a particular colony suggested greater pathogenicity to the germinating seeds than others, then this colony was

selected for isolation.

Screening tests for pathogenicity were conducted in 200 mm. test tubes as shown in Figure 3. Absorbent cotton moistened with distilled water, was placed at the bottom of each tube. The tubes were then plugged with cotton and sterilized at 15 pounds pressure for 20 minutes. A vacuum counter capable of picking up 5 clover seeds at a time (Figure 3), was employed to place surface disinfected seed in the sterilized tubes. Seed was surface disinfected as described in Chapter III. Initially six replicates of 5 seeds per tube were used, later this procedure was modified to three replicates of 10 seeds per tube. The seeded test tubes were incubated at 21°C and kept continuously illuminated by four 20 Watt fluorescent lamps (General Electric - "Cool White") as shown in Figure 4. When the seedlings were from 3 to 5 days old they were inoculated with mycelial fragments cut from the perimeter of 10 to 14 day old colonies grown on malt extract agar. The amount of damping-off and seedling decay was recorded ten days after inoculation (Figure 5).

The first pathogenicity screening trials were replicated on crimson, red, alsike and ladino clovers. As there was little evidence of susceptability differences among the four clovers, later tests were limited to red clover alone.

Pathogenicity tests in soil involved inoculation of surface disinfected seed (see Chapter III for disinfecting procedure) with fungi selected for their high degree of virulence during the preceding screening tests. The inoculated seed was sown in four, 80

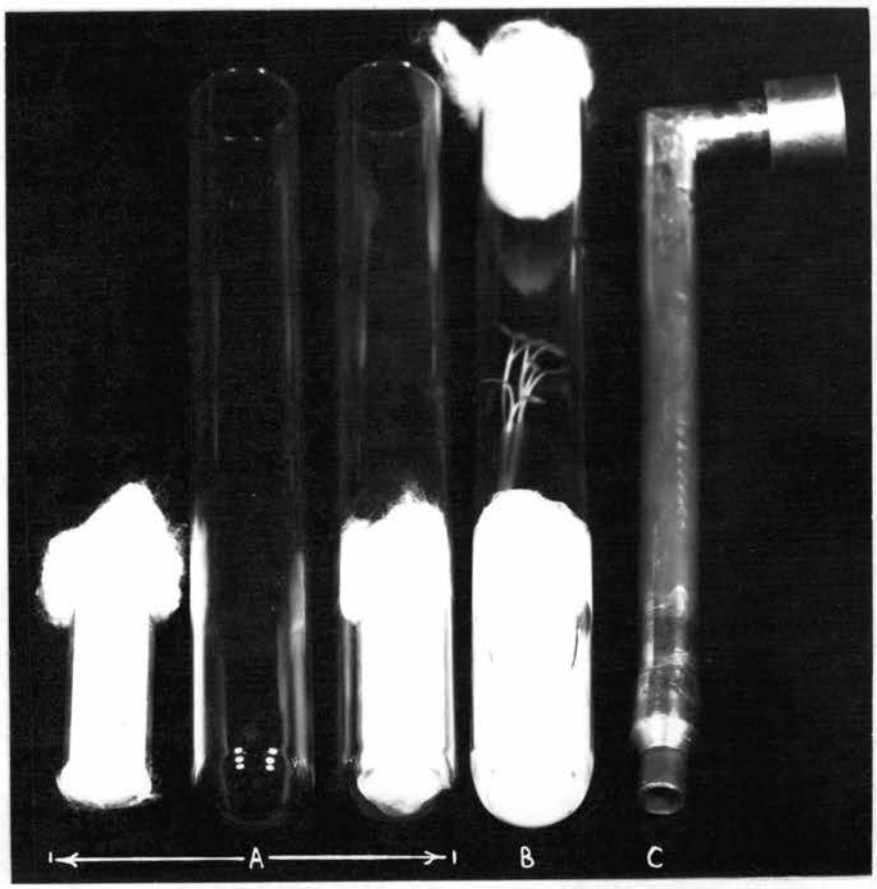

Figure 3. Procedure for growing aseptic seedlings in test tubes.
A) Method of preparing test tubes prior to seeding.
B) Seeded test tube with aseptic seedlings.
C)Vacuum counter employed for picking up 5 clover seeds at a time and dropping them into test tubes.

Figure 4. Pathogenicity screening tests on clover seedlings kept under continuous illumination at 21°C. (Photo. by H. H. Millsap)

Figure 5. Crimson clover seedlings five days after inoculating with fungi isolated from clover seed. Control (left). Inoculated with Ascochyta imperfecta (center). Inoculated with Sclerotinia sp. (right). (Photo. by H. H. Millsap)

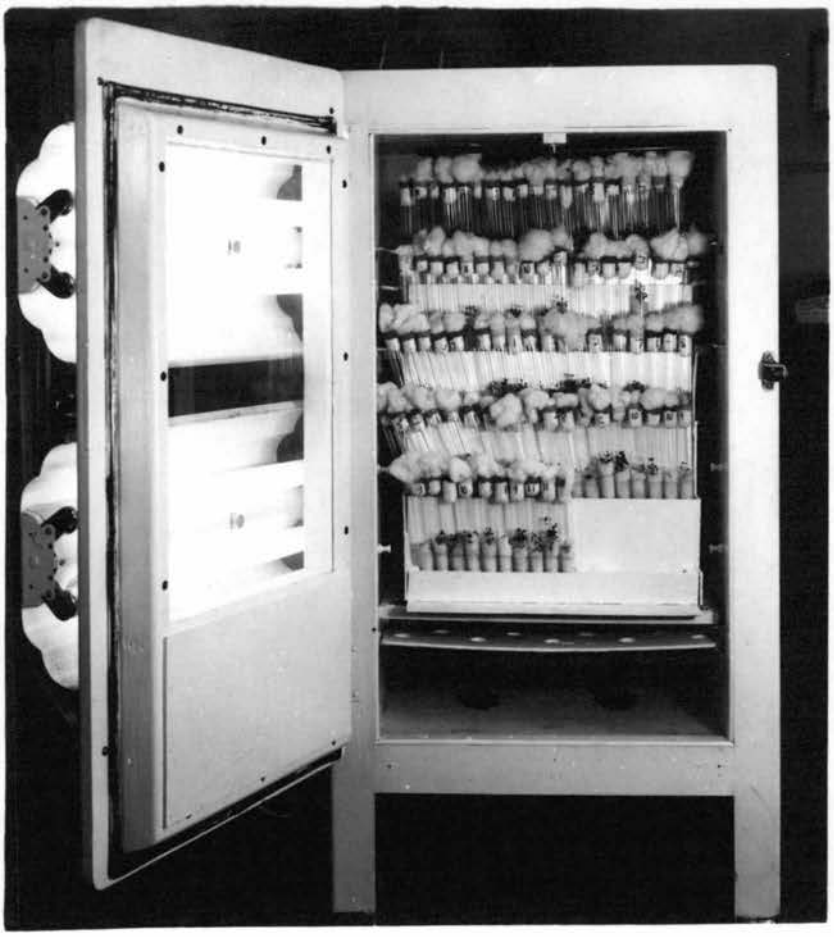

Figure 4.

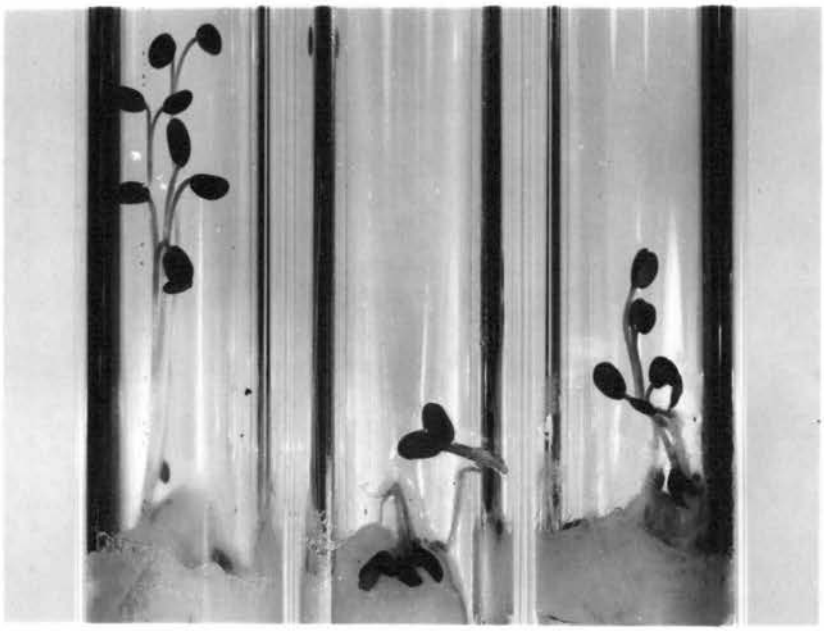

Figure 5.

seed replicates. Fungus inoculum was prepared by placing two 10-14 day old cultures grown on malt extract agar in a "Waring blender" with 500 ml. of distilled water and comminuting for one minute. The seed was sown in rows four inches apart in steam sterilized flats of a sandy loam soil. Inoculation was accomplished by pouring 100 ml. of inoculum over the exposed seed in the furrows. After inoculation the seed was covered with soil to a depth of one quarter inch. Data were taken twenty days after sowing. The seedlings were counted and examined individually (Figure 6). Each seedling was rated as healthy, slightly necrotic, or severely necrotic (Figure 7). Those rated slightly necrotic, were any having necrotic spots which failed to girdle the primary root or hypocotyl. Severely necrotic seedlings were completely girdled with little chance of survival.

Tests were also made in flats of soil to determine the influence of depth of planting on pathogenicity of the fungi. Inoculation was accomplished by dipping approximately 2 cc. of the seed, lightly wrapped in cheese cloth into comminuted inoculum. The effectiveness of this procedure is shown in Figure 8 where the inoculated fungi may be observed uniformly growing from plated seed. After inoculation, the seeds were allowed to dry in partly opened petri dishes. When thoroughly dry, 600 seeds were counted into 6 replicates of 100 seeds each, and sown in steam sterilized flats of a sandy loam soil. Of the six replicates inoculated with each organism, 3 were sown at a depth of one quarter of an inch and 3 at a depth of three quarters of an inch. Data were taken 17 days

Figure 6. Pathogenicity test in soil showing damping-off of 12 day old red clover seedlings grown from inoculated seeds. Each row was sown with 80 seeds. (Photo. by H. H. Millsap).

later according to the method described above.

Pathogenicity tests on the roots of mature plants were accomplished by transplanting four-month-old field-grown red clover plants into steam sterilized, sandy loam in the greenhouse. Each of the fungi selected from the preceding trials was inoculated onto 10 red clover plants with tap roots of one quarter of an inch to three quarters of an inch in diameter. Inoculation was accomplished prior to transplanting by a combination of root pruning, hammering of the root ends, cutting pieces three quarters of an inch in length off the side of the roots just below the first node, and then dipping the roots into the inoculum. The inoculum was prepared by the method

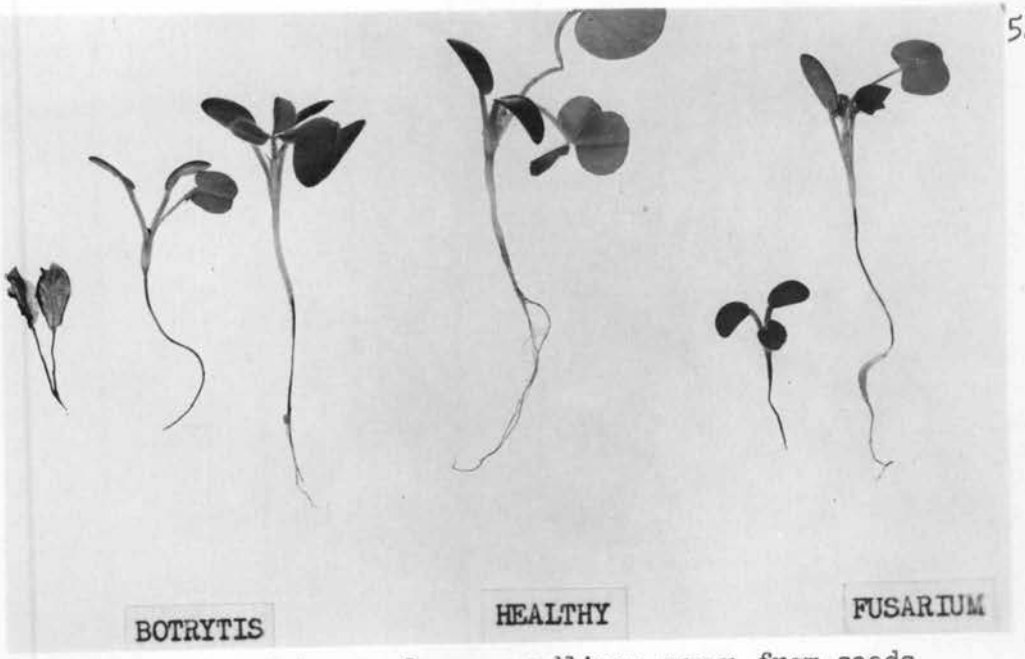

Figure 7. Crimson clover seedlings grown from seeds inoculated with fungi (fungi originally isolated from clover seed).

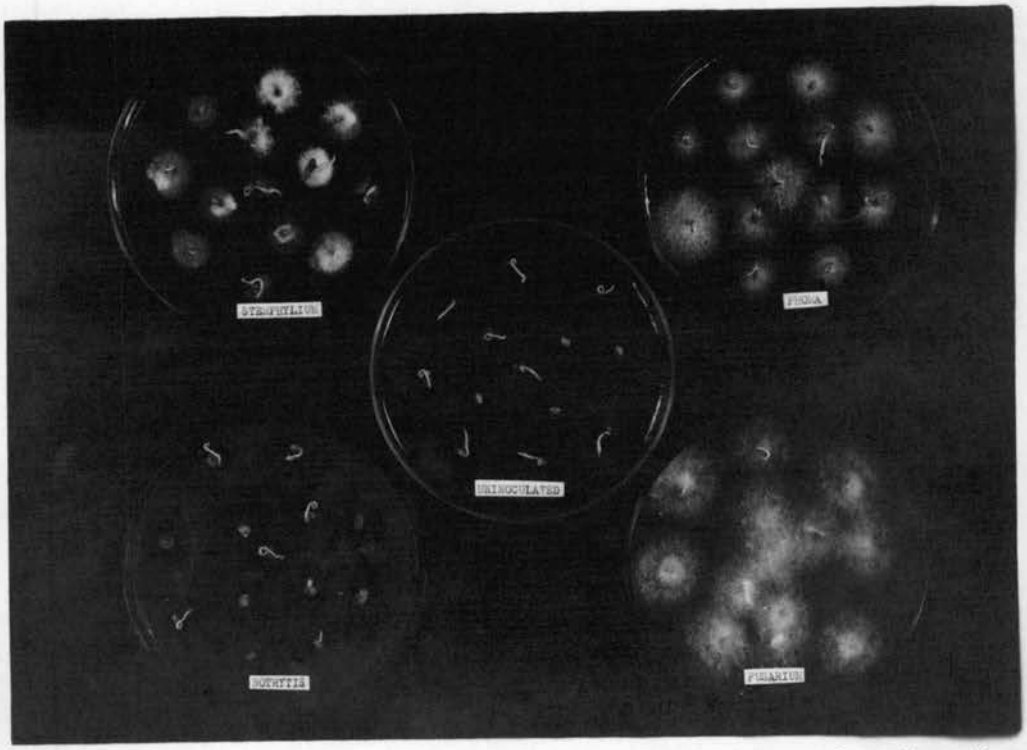

Figure 8. Plated clover seeds showing the uniformity of inoculum on seeds artificially inoculated with Stemphylium botryosum, Phoma trifolii, Botrytis cinerea and Fusarium roseum.

previously described. One hundred milliliters of inoculum was poured around each plant before it was tamped into the steam sterilized soil. Roots of mature plants were also inoculated in the field by wounding the tap root with a scalpel and then pouring 100 ml. of inoculum around the crown. Each of the fungi tested, was inoculated onto 10 plants. In both the greenhouse and field tests, the plants were removed eighty days after inoculating, washed and examined for root infections. Isolations were made from plants showing necrotic and discolored cortical or vascular tissue. The isolations were made by surface sterilizing with a fresh mixture of sodium hypochlorite and ethanol, and plating pieces of root onto malt extract agar.

Several miscellaneous pathogenicity trials were attempted. Two isolates of unknown species of Verticillium from clover seed were inoculated onto tomato (Lycopersicon esculentum var. "Bonny Best") and pepper (Capsicum frutescens var. "Red Cayenne"). One month old plants were root pruned, dipped into comminuted fungus colonies and planted in three replicates of 5 plants, in a steam sterilized, sandy loam soil. The plants were harvested and examined six weeks after inoculation.

Botrytis cinerea was the only fungus tested as a foliar parasite. Six-week-old red clover plants were grown in three replicates of two plants, at relative humidities ranging between 70-80 per cent and temperatures ranging from 75° to 98°F. Inoculation was by spraying the foliage with comminuted cultures. The foliage was kept moistened by spraying twice daily with distilled water. Results were taken

ten days after inoculation.

Results of pathogenicity tests

Results of the pathogenicity screening tests, and tests on inoculated seed sown in soil, are presented in Tables IV, V and VI, and in Figures 9 and 10. Individual fungi are treated independently in the following discussion.

Ascochyta imperfecta - caused considerable damping-off in screening tests and in tests with inoculated seed in soil (Tables V and VI, and Figure 9). Three isolates when inoculated onto seed and sown in sterile soil, caused damping-off or necrosis of 37, 61 and 79 per cent of the seedlings (Table VI). In pathogenicity tests on the roots of mature red clover plants, A. imperfecta was reisolated only infrequently. The weak pathogenicity on clover roots was to be expected, for A. imperfecta is regarded as a foliar pathogen rather than a root parasite.

Botrytis cinerea - All isolates of this fungus caused rapid damping-off in the screening tests (Table V), and in tests on inoculated seed sown in soil (Figure 9). The two isolates used in this latter test caused damping-off or necrosis (Figure 7) of 75 and 93 per cent respectively of the seedlings (Table VI). Foliar pathogenicity tests showed that B. cinerea was capable of causing severe damage to the foliage of clover under warm humid conditions. Root inoculations of mature plants were negative both in the field and in the greenhouse.

B. cinerea was also observed to be one of the most destructive

Table IV. Pathogenicity screening tests employing seedlings of Trifolium incarnatum, T. repens T. hybridum, and T. pratense inoculated with Fusarium roseum.

Number of isolate	Trifolium species tested							
	T. pratense		T. incarnatum		T. hybridum		T. repens	
	Seedlings inoculated	Seedlings killed	Seedlings inoculated	Seedlings killed	Seedlings inoculated	Seedlings killed	Seedlings inoculated	Seedlings killed
	Number	Per Cent	Number	Per Cent	Number	Per Cent	Number	Per Cent
98 NS(L)	17[1]	76[2]	25	100	22	100	13	84
72 NS(R)	21	100	22	95	22	100	16	56
44 SS(C)	24	100	17	94	23	87	24	100
90 NS(L)	23	39	26	92	21	52	19	53
45 NS(C)	19	0	25	4	25	36	16	0
78 NS(L)	19	89	23	35	25	28	19	84
88 NS(R)	21	24	21	10	23	4	18	0
110 NS(A)	21	0	23	0	26	4	18	6
98 NS1(L)	15	7	29	24	25	0	13	15
89 NS(R)	21	5	26	0	22	0	12	8
44 SS1(C)	25	0	28	4	23	0	12	0

[1] Total number of seeds germinating in 3 replications (10 seeds per rep.).
[2] Percentage of inoculated seedlings killed.

Table V. Pathogenicity screening tests on red clover seedlings inoculated with fungi isolated from clover seeds.

Fungus	Isolate Number	Seedlings inoculated	Seedlings killed
		Number	Per Cent
Alternaria tenuis	124NS(A)	22[1]	45[2]
	46NS(C)	22	0
	77NS(L)	20	75
	2824B(R)	25	4
	114NS(A)	25	4
	122NS(A)	21	0
	44SS(C)	22	18
Ascochyta imperfecta	48SS(C)	24	67
	18714NS(C)	19	47
	50NS(C)	17	47
	50SS(C)	21	48
	18037NS(C)	21	48
	98NS1(L)	20	20
Botrytis cinerea	4540NS(C)	21	71
	46NS(C)	23	91
	18371SS(C)	22	86
	118NS(A)	19	95
	2824(R)	25	72
Phoma trifolii	90SS(L)	16	13
	44NS(C)	22	36
	4540NS(C)	22	73
	4540NS2(C)	16	13
	127NS(A)	17	6
	113NS(A)	15	27
	46NS(C)	18	11
	4545(C)	18	72
	47SS(C)	19	16
	1212SS(C)	21	5
Stemphylium (Pseudoplea sp.?)	74NS1(L)	17	6
	74NS2(L)	19	42
	74NS3(L)	16	75

(continued on Page 59)

Table V. (continued) Pathogenicity screening tests on red clover seedlings inoculated with fungi isolated from clover seeds.

Fungus	Isolate Number	Seedlings inoculated	Seedlings killed
		Number	Per Cent
Stemphylium consortiale	83SS(R)	14	0
	122NS(A)	19	0
	127NS(A)	20	0
	118NS(A)	21	0
	76NS(L)	18	11
	45SS(C)	15	0
	44NS(C)	22	14
Stemphylium botryosum	107SS(A)	21	5
	124NS(A)	19	5
	47NS(C)	23	57
	44SS(C)	22	36
	45SS(C)	22	77
Nigrospora oryzae	69SS(R)	19	0
Verticillium sp.	101NS(L)	21	0
	46(C)	20	5
	84SS(R)	24	0
	70NS(R)	21	0
Check		21	0

[1] Total number of seeds germinating from 3 replications (10 seeds per rep.).
[2] Percentage of inoculated seedlings killed.

fungi encountered on germination test blotters, where it spread fairly rapidly causing damping-off. The fungus was readily identified from the growth of conidia and sclerotia; the latter usually being produced in the dead seedling tissue within 10 days of initial infection.

Cladosporium cladosporioides - caused necroses and sometimes death of red clover seedlings in blotter germination tests[5], and in pathogenicity screening tests. Attacked hypocotyls showed brown lesions upon which the fungus fruited profusely. Though this fungus was almost universally present in Oregon clover seed, pathogenic tendencies appeared to be restricted mainly to seed from the Willamette Valley that had been harvested under wet conditions.

To the writer's knowledge, C. cladosporioides has not been reported as a pathogen of Trifolium spp. in the field. Greenhouse tests in which seeds heavily infested with this fungus were sown untreated and treated with the fungicide "Arasan" in sterilized and unsterilized soil, failed to show significant reduction of germination as a result of this fungus (Table XIII, Appendix B). It is concluded from limited data, that under certain circumstances C. cladosporioides may damage seedlings in germination tests, but it is unlikely that it significantly affects germination of clover seeds in the field.

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[5]This condition was reported to the writer by seed-analysts of the state seed testing laboratory, and of E. F. Burlingham and Sons. It was first observed by these analysts in seed harvested in 1953 and 1954.

Fusarium roseum - varied considerably in pathogenicity from isolate to isolate. In the screening tests (Table IV) some isolates were extremely virulent, rapidly killing inoculated seedlings, whereas others were only weakly parasitic. The four most virulent isolates selected from the screening test, when inoculated onto seed, which in turn was sown in soil, caused only 10 per cent necrosis or damping-off (Table VI). However, by sowing inoculated seed at three quarters of an inch depth rather than at one quarter of an inch, the number of necrotic or damped-off plants was increased from 9 per cent to 62 per cent for one Fusarium isolate (Figure 10). In pathogenicity tests on the roots of mature plants, negative results were obtained.

Phoma trifolii - isolates varied considerably in virulence in all trials (Table V and VI, and Figure 9). Under the conditions of the screening tests some isolates were weakly parasitic whereas a few were strongly pathogenic. Three isolates selected from the screening tests, when inoculated on seed in sterile soil, caused damping-off or necrosis of seedlings amounting to 6, 16 and 85 per cent (Table VI, and Figure 9). When P. trifolii was inoculated into the roots of mature red clover plants, it was reisolated only infrequently. As P. trifolii is mainly a foliar parasite of clovers, a high degree of pathogenicity on roots was not expected in root inoculations.

Stemphylium sp. (Pseudoplea?) - Isolates caused considerable damping-off of seedlings in the screening tests. Inoculated seed sown in sterile soil was not significantly affected (Tables V and VI, and Figure 9).

Sclerotinia sp.- caused rapid damping-off of inoculated seedlings during screening tests but showed only weak pathogenicity on seeds sown in sterile soil (Figure 9). Roots of mature plants inoculated with this fungus were not affected.

Stemphylium botryosum - Isolates of S. botryosum were mainly weak pathogens under the conditions of the trials. Certain isolates however, caused considerable damping-off in the screening tests (Table V) and in the tests in which inoculated seed was sown in soil (Figure 9). One isolate caused damping-off or necrosis of 47 per cent of the seedlings, when inoculated seed was sown in sterile soil (Table VI). Pathogenicity tests on roots of mature plants were negative.

Verticillium sp. - showed little tendency to parasitize clover seedlings (Table V). When inoculated into the roots of mature red clover plants, one isolate of Verticillium (Isolate no. 101SS) was consistently reisolated from the vascular tissue of the primary root. Five week old crimson clover plants planted in soil infested with Verticillium (Isolate no. 72NS), were significantly smaller than check plants when harvested after 11 weeks. The 3 replicates of 5 plants from the infested soil weighed an average of 50 per cent lighter than the checks. Verticillium was consistently reisolated from these plants. None of the Verticillium isolates was pathogenic when inoculated onto tomatoes (Lycopersicon esculentum var. "Bonny Best") or pepper (Capsicum frutescens var. "Red Cayenne") which

Table VI. Pathogenicity of suspected pathogenic fungi inoculated onto red clover seed, which was in turn sown in flats of sterilized soil (Fungi originally isolated from clover seed).

Fungus	Culture no.	Infected and damped-off seedlings
		Per Cent[1]
Botrytis cinerea	46NS(C)	75
	2824(R)	93
Stemphylium botryosum	47NS(C)	3
	44SS(C)	4
	45SS(C)	47
Ascochyta imperfecta	48SS(C)	37
	18714NS(C)	61
	50SS(C)	79
Phoma trifolii	44NS(C)	6
	4540NS(C)	16
	4545(C)	85
Stemphylium sp. (Pseudoplea?)	74NS(L)	0
Alternaria tenuis	77NS(L)	6
Sclerotinia sp.	18371SS(C)	3
	4540SS(C)	16
Fusarium roseum	98NS1(L)	9
	72NS(R)	10
	44SS2(R)	10
	78NS	13
Control		0

[1] Percentage of infected and damped-off seedlings relative to the control (averaged from four replicates).

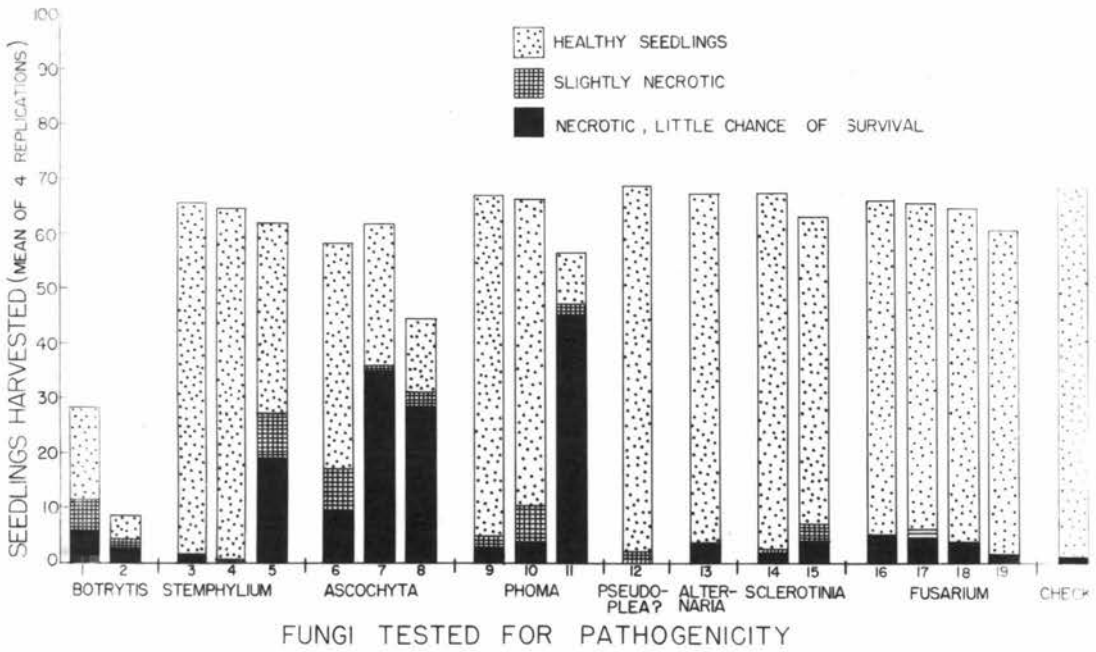

Figure 9. Pathogenicity of suspected pathogenic fungi inoculated onto red clover seed, which was in turn sown in flats of sterilized soil. (The numbers at the base of each bar indicate the different isolates of Botrytis cinerea, Stemphylium botryosum, Ascochyta imperfecta, Phoma trifolii, Stemphylium sp. (Pseudoplea?), Alternaria tenuis, Sclerotinia sp., and Fusarium roseum tested.)

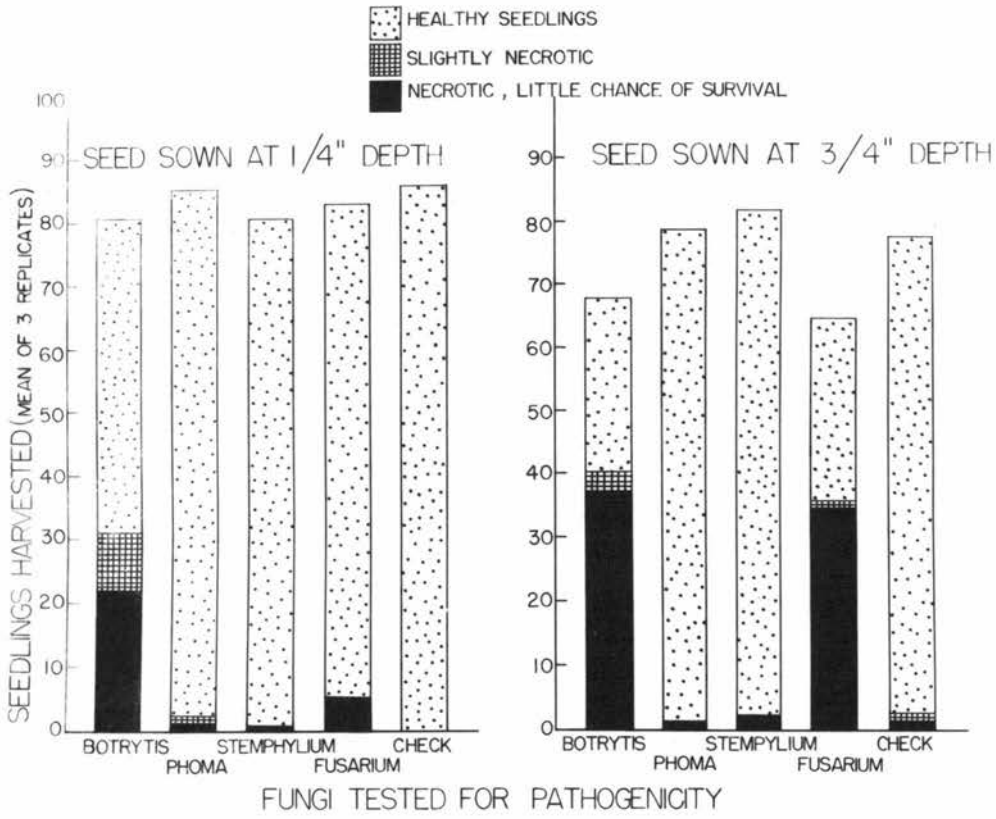

Figure 10. The influence of depth of sowing on the emergence of seedlings from seeds inoculated with Botrytis cinerea, Phoma trifolii, Stemphylium botryosum and Fusarium roseum (Isolates 46NS, 44NS, 44SS, and 45SS respectively).

suggested that the species were probably neither V. dahliae or V. albo-atrum.

Other miscellaneous fungi were screened for pathogenicity (Table V). Certain isolates of Stemphylum consortiale were very weak pathogens on seedlings. Nigrospora oryzae showed no pathogenic tendencies. Isolates of Alternaria tenuis showed little pathogenisis, except for two isolates which caused considerable damping-off in screening tests (Table V), but which lacked this virulence in soil tests (Figure 9).

Discussion

Because of the limited time available for this study, it was not possible to carry out the various pathogenicity tests under different environmental conditions. Thus where certain isolates were apparently not pathogenic, this failure may have been due to the unfavorable environmental conditions of the test. Indeed it was shown that depth of sowing alone could significantly influence the amount of damping-off caused by Fusarium roseum and Botrytis cinerea (Figure 10). It is also likely that the optimum temperature for pathogenicity of some of the fungi, was different from those employed in the tests. With allowance for these deficiencies, the results indicate that a number of fungi carried on Oregon grown clover seed are pathogenic at least to the point of causing damping-off of clover seedlings. Whether naturally infested seeds act as centers of infection from which healthy plants might become infected, was not determined; nevertheless this possibility remains. If 0.5 per cent of the seed of a red

clover sample were infested for example with Phoma trifolii, and this seed were sown at the rate of 16 pounds to the acre (1 oz. of red clover seed contains approx. 17,010 seeds), then 21,777 seeds would carry the fungus. Similarly a sample with a 25 per cent infestation of seed, would result in one million contaminated seeds being sown. Bearing in mind that seed examined during the survey was often infested by pathogenic fungi to the extent of 0.5 to 20 per cent or more, it would seem likely that such seed when sown in the field could initiate foliar infection under suitable environmental conditions.

Pathogenicity studies in which the roots of mature plants were inoculated were generally inconclusive except for tests with two unidentified Verticillium isolates (see Appendix A). The constancy with which Verticillium was reisolated from inoculated plants, indicated that it was capable of acting as a pathogen. This is a significant finding for there are few records of seed transmission of species of Verticillium (Chapter II).

Finally, though pathogenesis of certain fungi associated with Oregon clover seed was proven in soil tests, it is difficult to interpret this from the standpoint of what happens when infested seed is planted in the field. Undoubtedly some damping-off of seeds infested with pathogens will occur in the field, though this in itself is probably of little importance, for clovers are usually sown at a heavier rate than is necessary. What is believed to be important, is the possibility of infested seed acting as centers from which infection may spread to healthy plants. The relationship

between seed infestation and field infection is complex because of the interacting factors between host, parasite and environment. To attempt to evaluate these relationships for the numerous pathogens encountered, would have been far beyond the scope of this study.

CHAPTER V

NATURE OF THE ASSOCIATION OF SEED-BORNE FUNGI WITH CLOVER SEED

Fungi may be carried by seeds on the seed coats, as spores, mycelia or fruiting bodies; within or beneath the seed coats as mycelia; and as fragments of infected plant tissue, or sclerotia, mixed with the seed. The position and form of fungi relative to the seed, to some extent determine the effectiveness of seed treatment, the possibility of detection by visual examination, and the extent to which the fungi may be cleaned from the seed during processing.

The object of the work described below was to determine the position and form in which fungi encountered in Chapter III, were carried by the seed. Because of limitations of time, anatomical studies on the 33 fungi and four clovers involved could not be undertaken. Data were obtained from visual observations, from comparative plating of untreated and surface disinfected seeds, and from culturing washings from seeds.

Surface-borne fungi, sclerotia and mycelial fragments were determined by seed examination under a stereoscopic microscope (10-30x). Fungi adhering tightly to the seed coats as spores or mycelia, or within the seed as mycelia, were established by plating the seeds on malt extract agar. One half of each sample studied was surface disinfected to determine fungi within the seed and the other half was left untreated as a basis for determining all the organisms present. The work was carried out in conjunction with the survey, and employed the sampling and culturing methods described earlier. Some

knowledge of the incidence of organisms carried loosely on the seed coats was obtained by washing 2.8 cc. of seed in a sterile screwcapped culture tube, with 10 ml. of sterile water and 5 drops of 5 per cent "Vatsol" wetting agent. The tube was shaken vigorously for one minute after which two 0.5 ml. and 0.1 ml. samples of washings were withdrawn with a sterile pipette and transferred separately into tubes containing 10 ml. melted malt extract agar at approximately 40°C. Agar and washings were mixed by rotating the tubes gently to prevent frothing, and were poured into sterilized petri dishes. The plates were incubated at 21°C. Seven to ten days later records were made of the species of fungi present. Washings were examined from a combined total of 18 samples of red, crimson, ladino and alsike clovers.

Results

Three fungi were visibly conspicuous on the seed coats, or mixed with the seed. Sclerotia[6] were infrequently observed mixed loosely with crimson clover seed. Usually they were about the same size as the seeds. A few of the sclerotia were plated on malt extract agar and the resultant colonies were typical of a Sclerotinia spp. However, because of the limited number of platings, it is not possible to state

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[6] Mrs. M. Ryman (Seed analyst with E. F. Burlingham, Forest Grove, Oregon) examined the permanent records of the state seed testing laboratory from 1943-46, and found sclerotia noted in samples of crimson, alsike and red clovers (mainly Willamette Valley seed). In examining the records from 1951-1954, the writer found sclerotia noted in a few samples of crimson clover, also grown in the Willamette Valley.

that all the sclerotia observed belonged to the genus Sclerotinia.

Dark hyphae were observed ramifying over the seed coats of all four clover species (Figure 11). These were determined to be Cladosporium cladosporioides. Conidiophores were produced readily when the seeds were placed on moist filter papers. The infestation of clover seed coats by C. cladosporioides was very similar to the published photographs (62; 102) of the mycelium of the "blackpatch" fungus on seeds.

Figure 11. Ladino clover seeds showing mycelia of Cladosporium cladosporioides on seed coats. Seed at lower right is uninfested. x37.

A number of samples of ladino clover bore small black sclerotia like bodies on the seed coats (Figure 12) similar to those produced in culture by the unidentified Stemphylium sp. (Pseudoplea? -

Appendix A). All the samples infested with these bodies were likewise infested with the unidentified Stemphylium. No attempt was made to culture from these sclerotia, and the association between them and the unidentified Stemphylium is based on circumstantial evidence.

Figure 12. Ladino clover seeds with small sclerotia or incipient fruiting bodies. Structures are believed to belong to an unidentified species of Stemphylium (see Appendix A). x37.

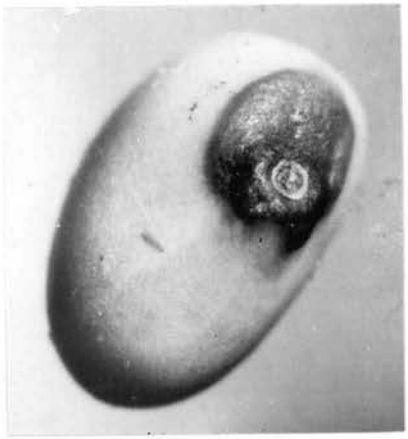

Figure 13. Crimson clover seed showing an area of decay centered on the hilum. x24.

In addition to the above fungi, visual examination of seed at magnification of 10-30X, often revealed decaying seeds. Spots of decay were commonly associated with the hilum (Figure 13), or with seed injuries; though some seeds without noticeable injury also showed decay. The decay frequently penetrated into the cotyledons and other seed parts causing death of the seed. No attempt was made to determine the fungi associated with the decayed areas.

The considerable reduction of microflora that always occurred on surface disinfecting seeds, (Table II, Chapter III) was taken to indicate that many organisms were confined to the seed surface and periferal regions of the seed coat where they were killed by the disinfectant. Surface disinfection of seeds however, rarely completely eradicated the seed-borne microflora. The inability of the disinfectant to kill all the fungi present, indicated that the surviving organisms had penetrated into the seed. Table VII shows that most of the fungi observed in Chapter III were able to penetrate, presumably in the form of mycelia, and thus escape the disinfectant. The degree of penetration by a particular species as suggested by its ability to survive surface disinfection, was found to vary widely between samples, e.g. in some samples infested with Cladosporium cladosporioides, surface disinfection of the seeds completely eradicated the fungus; whereas in others, using the same procedures, only a relatively small reduction resulted. Many of the samples in which the seeds showed deep penetration by fungi, originated from regions where the seed had been harvested under moist conditions.

It was noted during examination of plated seeds that seed coats were sometimes completely overgrown with fungi, yet unblemished radicals and cotyledons were able to emerge. It appeared as if the seed coats could support luxuriant growth of a number of fungi, without these fungi penetrating deeper into the seed.

Culturing from seed washings, revealed that many of the organisms associated with clover seed could be carried loosely on the surface of the seed as mycelia or as spores (Table VIII). Over 16 different species, including a species of Trichoderma not recorded during the survey, were observed from the 18 samples of seed examined. The frequency of occurrence of specific organisms from the seed washings paralleled those found from plating untreated seed in Chapter III.

Table VII compares the fungi found on surface disinfected and untreated seeds, and in seed washings. Seventy-five per cent of the species observed on untreated seeds were also present with surface disinfected seeds, compared to fifty-four per cent of the species cultured from washings. From these data it appears that many of the fungi observed during the survey were able to be present both as mycelia within the seed and loosely on the seed coats as spores or mycelia.

Table VII. Comparison of the fungi found on untreated and surface disinfected clover seeds, and in seed washings.

Fungus	Untreated seeds[1]	Treated seeds	Seed washings[2]
Alternaria tenuis	X[3]	X	X
Ascochyta imperfecta	X	X	X
Aspergillus spp.	X	X	X
Botrytis cinerea	X	X	X
Cephalosporium sp.	X	-	-
Cephalothecium sp.	X	-	-
Chaetomium cochliodes	X	-	-
C. funicola	X	-	-
Cladosporium cladosporioides	X	X	X
C. elatum	X	X	X
Didymium difforme (Myxomycete)	X	-	-
Epicoccum sp.	X	X	-
Fusarium roseum	X	X	X
Mucoraceous spp.	X	X	X
Nigrospora oryzae	X	-	-
Penicillium sp.	X	X	X
Phoma trifolii	X	X	-
Pullularia sp.	X	X	-
Sclerotinia sp.	X	X	-
Scopulariopsis brevicaulis	X	-	X
Stemphylium consortiale	X	X	X
S. botryosum	X	X	-
Stemphylium sp. (Pseudoplea?)	X	X	-
Verticillium sp.	X	X	X
White bacterium	X	X	X
Yellow bacterium	X	X	X

[1] 78 samples of seed examined (See Table II for more detailed information).
[2] 18 samples of seed examined (See Table VIII for more detailed information).
[3] X indicates the presence of the fungus.

Table VIII. Occurrence of loosely borne fungi on the surface of clover seeds as determined from culturing seed washings on malt extract agar.

Fungus	Number of samples infested[1]
Cladosporium cladosporioides	17
Bacterial spp.	14
Penicillium spp.	11
Alternaria tenuis	10
Mucoraceous spp.	7
Cladosporium elatum	6
Unknown spp.	4
Verticillium sp.	3
Aspergillus spp.	3
Stemphylium consortiale	2
Rhizopus sp.	2
Trichoderma sp.	2
Botrytis cinerea	1
Fusarium roseum	1
Ascochyta imperfecta	1
Scopulariopsis brevicaulis	1

[1] Eighteen clover samples were examined. (Five were Trifolium pratense, three T. repens, six T. incarnatum, and four T. hybridum).

CHAPTER VI

COMPARISON OF THE FUNGI FOUND IN THE AIR OVER A CLOVER FIELD, WITH THOSE ASSOCIATED WITH SEED HARVESTED FROM THE SAME FIELD

Many of the fungi observed on clover seed were suspected of being saprophytic, air-borne molds capable of contaminating seed in the field or during harvesting and cleaning. Clarification of this relationship was sought by sampling fungi in the air over a three acre crimson clover field and comparing the results with the fungi found on seed harvested from the same field.

The spore traps employed were simply lids of 90 mm. diameter petri dishes containing 15 ml. of sterilized paraffin oil. The dishes were covered with discs of 60 mesh copper screen to reduce contamination of the paraffin oil by insects, grass anthers, and other extraneous matter. The screen discs were cut about 15 mm. wider than the diameter of the dishes. The edges were bent down and crimped about every 4 cm. to hold the screens firmly in place. Three traps were placed at each location at distances of one inch, twenty-one inches and sixty inches above the ground. Each trap was placed in a roofed galvanised platform (Figure 14) attached to a 2" x 2" post. Three of these posts accommodating a total of nine traps, were placed in well separated positions through the clover field.

The first traps were exposed when the petals of most of the crimson clover flowers had withered but had not yet dropped. After four days, the traps were removed, the screens thoroughly washed in

95 per cent ethyl alcohol and replaced on fresh traps. This procedure was repeated every four days until harvest.

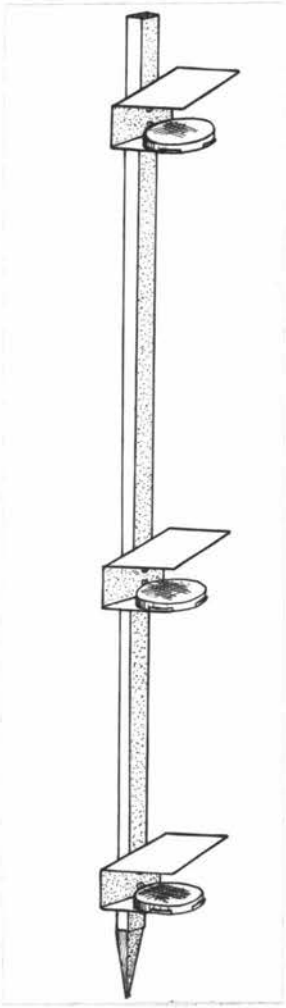

Figure 14. Arrangement of spore traps at each sampling location.

Spores were determined by pouring the oil from the traps into centrifuge tubes containing 1 ml. of sterile water, and centrifuging at 3,000 r.p.m. for 15 minutes. After centrifuging, 0.5 ml. of water and sediment was removed from the bottom of each centrifuged tube with a sterile pipette, and placed separately in screw capped culture tubes containing 15 ml. of liquid malt agar cooled to approximately 40°C (Duplicate tubes were prepared). The contents of the tubes were mixed by gently rotating, pouring into sterilized petri dishes and

incubating at 21°C for 7 to 10 days. After incubation, the fungus colonies growing on the malt agar were identified and recorded.

After the seed from the field in which the spore traps had been placed was harvested and cleaned, each 100 pound sack was sampled with a "thief" type trier. The samples were mixed for uniformity with a "Gamet" divider and 300 untreated seeds were plated on malt agar. Fungi were determined following the methods described in Chapter III.

Results

More than fourteen species were found in the air over the crimson clover field (Table XI). All but one of these, a pink bacterium, had been found on clover seed during the survey work reported in Chapter III. Eighty-two per cent of the species isolated from seed of this field, were also present in the air traps.

Neither the weather during the air sampling period, nor the height of the spore traps above the ground, seemed to effect the composition of micro-organisms found in the traps significantly (Table X). Many of the species found at flowering were also present at harvest.

Although this experiment was limited to one clover species in a single location, it did show that most of the fungi associated with the crimson clover seed were abundantly present in the air over the field.

Many of the organisms observed in the spore traps are saprophytes of widespread occurrence that are commonly present in soil

and air.

Table XI. Comparison of micro-organisms trapped in the air above a field of crimson clover, with those present on seeds harvested from the same field.

Fungus	Air traps	Seed
Alternaria tenuis	X[1]	X
Ascochyta sp.	X	-
Aspergillus spp.	X	-
Bacterium (white)	X	X
Bacterium (pink)	X	-
Botrytis cinerea	X	X
Cephalothecium sp.	-	X
Cladosporium cladosporioides	X	X
Epicoccum sp.	X	X
Fusarium sp.	X	X
Mucoraceous spp.	X	X
Penicillium spp.	X	X
Pullularia sp.	X	-
Stemphylium consortiale	-	X
S. botryosum	X	X
Sterile mycelia	X	-
Verticillium sp.	X	-

[1] X indicates the presence of the fungus.

Table X. Organisms collected from spore traps located in a field of crimson clover during a period of one month prior to harvesting seed.

Organism	Trap height	Number of traps in which micro-organisms occurred. June 23	June 27	July 1	July 5	July 9	July 13	July 18	July 22	total no. of infested traps
	Inches	Number	Number	Number	Number	Number	Number	Number	Number	Number
Alternaria tenuis	60[1]	1[2]	-	2	2	-	2	2	1	
	21	1	-	1	2	1	1	2	1	
	1	-	-	-	1	-	1	1	1	23
Ascochyta sp.	60	-	-	-	-	-	-	1	-	
	21	-	-	-	-	-	-	-	-	
	1	-	-	-	-	-	-	-	-	1
Aspergillus sp.	60	-	-	-	-	-	-	-	-	
	21	-	-	-	-	-	-	-	-	
	1	-	-	-	-	1	-	-	-	1
Bacteria (white)	60	2	-	2	3	1	-	3	-	
	21	2	1	2	2	2	-	1	1	
	1	2	-	2	1	2	-	2	1	32
Bacteria (pink)	60	2	-	-	1	-	-	2	-	
	21	2	1	-	-	-	-	1	1	
	1	2	-	-	1	-	-	2	1	16
Botrytis cinerea	60	-	-	2	1	1	-	-	-	
	21	-	-	-	1	1	-	-	1	
	1	-	-	-	-	-	1	-	1	9

(continued on Page 82)

Table X. (continued) Organisms collected from spore traps located in a field of crimson clover during a period of one month prior to harvesting seed.

Organism	Trap height	Number of traps in which micro-organisms occurred. June 23	June 27	July 1	July 5	July 9	July 13	July 18	July 22	Total no. of infested traps
	Inches	Number	Number	Number	Number	Number	Number	Number	Number	Number
Cladosporium cladosporioides	60	2	2	3	1	3	3	2	2	
	21	3	3	2	3	3	2	3	1	
	1	3	2	2	2	1	2	3	2	55
Eppicoccum sp.	60	-	-	-	-	-	-	1	1	
	21	-	-	-	-	-	1	1	-	
	1	-	-	-	-	-	-	-	-	4
Fusarium sp.	60	1	-	-	-	-	-	-	-	
	21	-	-	-	-	-	-	-	-	
	1	1	-	-	-	-	1	-	-	3
Mucor spp.	60	-	-	-	-	1	-	-	-	
	21	1	-	1	1	1	-	-	-	
	1	-	-	-	-	-	1	-	-	6
Penicillium spp.	60	-	-	1	-	-	2	1	1	
	21	1	2	1	-	-	-	1	-	
	1	3	-	1	-	1	-	-	1	16
Pullularia sp.	60	-	-	-	-	-	1	1	-	
	21	-	-	1	-	1	1	1	-	
	1	1	-	-	1	1	3	-	1	13

(continued on Page 83)

Table X. (continued) Organisms collected from spore traps located in a field of crimson clover during a period of one month prior to harvesting seed.

Organism	Trap height	Number of traps in which micro-organisms occurred. June 23	June 27	July 1	July 5	July 9	July 13	July 18	July 22	Total no. of infested traps
	Inches	Number	Number	Number	Number	Number	Number	Number	Number	Number
Stemphylium botryosum	60	-	-	-	-	-	2	-	-	
	21	-	-	1	-	-	1	-	1	
	1	-	-	-	-	-	-	1	1	7
Sterile mycelia	60	-	-	-	-	-	1	-	-	
	21	1	-	-	-	-	1	1	1	
	1	1	-	-	-	-	2	-	-	8
Verticillium sp.	60	-	-	-	-	-	-	1	1	
	21	-	-	-	-	-	-	-	-	
	1	-	-	-	-	-	-	-	1	3

[1]Traps were replicated 3 times at each height.
[2]Number of traps infested with a specific organism.

CHAPTER VII

THE RESPIRATION OF THE SEED-BORNE MICROFLORA OF GERMINATING ALSIKE CLOVER SEEDS

The fact was brought out during earlier phases of this investigation, that many of the micro-organisms associated with clover seed were in intimate contact with the seed coats. This prompted the writer to wonder whether or not the respiration of the microflora was a significant part of the total respiration of germinating seeds. If this were the case, respiration measurements might provide a method for rapidly determining the degree of infestation of seed.

Respiration rate of alsike clover seed, known from the survey to be heavily infested with molds, was studied in a Warburg respirometer. After a number of preliminary trials, the following tests were made.

Treatment	Purpose of treatment
1. Untreated seed	To determine total respiration of the seed and microflora.
2. Surface disinfected seed	To determine respiration of the seed only.
3. Surface disinfected seeds plus washings from untreated seed.	To determine whether or not the washings would account for some of the difference between 1 and 2.
4. Triturated[7], surface disinfected seed.	To determine whether triturated seed would show any respiration.

(continued on next page)

[7] The respiratory activity of the seed was destroyed by trituration in a mortar, followed by several days storage in a sterile container. Trituration did not noticeably effect the respiration of the seed microflora.

Treatment	Purpose of treatment
5. Triturated, untreated seed.	To determine the respiration of micro-flora alone, in the presence of the triturated seed.
6. Triturated, surface disinfected seed plus washings from untreated seed.	To determine whether the washings would account for the difference between 4 and 5.
7. Washings alone from untreated seed.	To determine if the micro-organisms could respire in the absence of seed.

The above treatments were duplicated in sterilized respirometer flasks as follows: a) 0.3 ml. of 5 per cent KOH and a small filter paper wick were added to the center wells to remove carbon dioxide. b) One milliliter of sterile water was added to the flasks to allow the seed to germinate (washings[8] replaced water in treatments 3, 6 and 7). c) 200 dry seeds either untreated, surface disinfected[9] or triturated, were added to the flasks except in treatment number four, where no seed was employed.

The flasks were kept in constant motion at 30°C. Seed was allowed to soak in the agitating flasks for 15 hours before the manometers were closed to the outside air. By this time, most seeds had developed radicals up to one quarter of an inch long. Manometer

[8]Seed washings were obtained from 200 untreated seeds shaken vigorously for one minute in 10 ml. of sterile water to which one drop of 5 per cent "Vatsol OT" was added. The washings were centrifuged at 3,000 r.p.m. for 15 minutes. One milliliter of the sediment was removed with a sterile pipette for use in the respirometer flasks. Washings obtained in this manner naturally were not equivalent of the total seed microflora, but only of the organisms loosely borne on the seed coats.

[9]Surface disinfection and drying of treated seeds are described in Chapter III.

readings were taken at thirty minute intervals over a period of five hours. The rate of respiration was obtained by plotting micro-liters of oxygen against time.[10]

Results

The rate of oxygen uptake of germinating, untreated seed (Figure 15) was greater than for surface disinfected seed.[11] Addition of seed washings to the surface disinfected seed resulted in an increase in oxygen up take, though a level equivalent to that of the untreated seed was not reached. The seed washings alone showed no measurable respiration.

Triturated, untreated seed had the highest rate of oxygen uptake of any of the treatments. Unlike the whole, untreated seed, this rate was not constant but increased with time. Triturated, surface disinfected seed to which seed washings had been added, behaved similarly to the triturated, untreated seed. Triturated surface disinfected seed alone, showed a very slow rate of oxygen uptake. The difference in rate of oxygen usage between untreated and surface disinfected seed indicated that the seed microflora contributed considerably to the overall respiration of the germinating alsike

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[10]For purposes of calculation the volume of 200 seeds used in the treatments was determined by placing 800 seeds in a 10 ml. graduated cylinder and then adding a known volume of ethanol with a pipette. The volume of ethanol, subtracted from the volume of seeds plus ethanol, gave the volume of 800 seeds; from which the volume of 200 seeds was obtained.

[11]The germination of surface disinfected seeds was identical with untreated seeds, as determined by germination tests.

clover seed. The increase in rate of respiration that occurred when seed washings were added to the surface disinfected seed was believed to be due to the micro-organisms in the washings, though this increase could be explained as a stimulatory effect on the respiration caused by the chemicals contained in the washings. Inactivation of seed by trituration clarified this situation, for it eliminated the seed as a living respiring entity, and allowed the respiration of the micro-organisms alone, to be considered. The triturated, surface disinfected seed showed a low rate of oxygen uptake. This was accounted for by the consideration that surface disinfection was not usually complete, (Table II) and fungi which had penetrated deeply into the seed coat were able to survive and to utilize a small amount of oxygen. The untreated, triturated seed and the surface disinfected, triturated seed to which seed washings had been added, utilized oxygen at a rate even greater than the rate of use by untreated seeds, indicating that trituration had made seed storage materials more readily available to the micro-organisms.

It is concluded that much of the total respiration of a sample of germinating alsike clover seed was attributable to the seed microflora. This evidence supports the earlier supposition that respiration measurements offer a possible means for rapidly determining the degree of seed infestation by molds. However, further work must be performed before the practicality of the method can be evaluated.

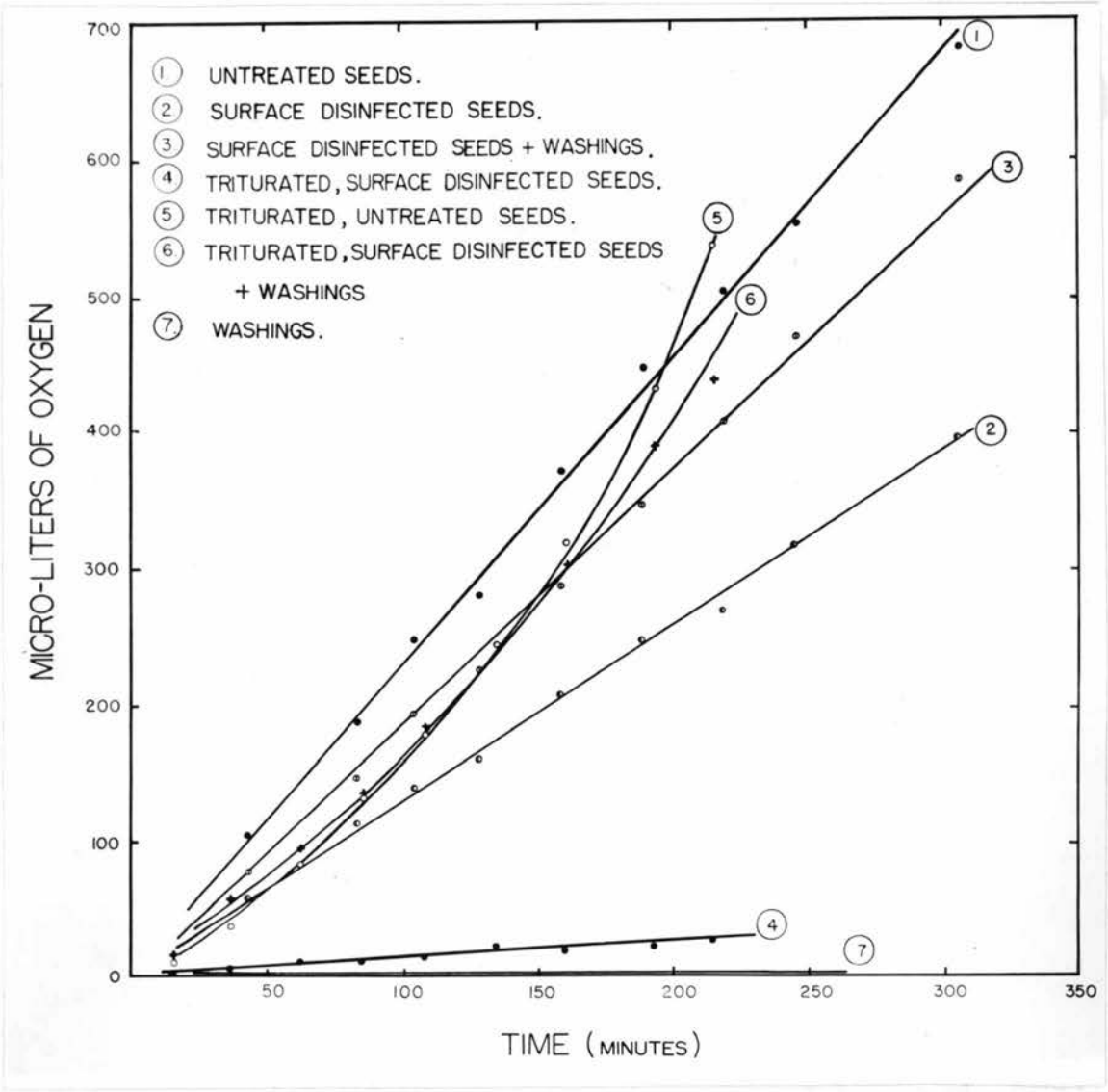

Figure 15. The relationship of the total respiration of germinating alsike clover seed to the respiration of surface disinfected seed, and to the seed microflora.

CHAPTER VIII

LONGEVITY OF MICRO-ORGANISMS ON CLOVER SEED STORED AT ROOM TEMPERATURE

Knowledge of the longevity of micro-organisms on seed is of interest, for in circumstances where seed-borne pathogens are viable for only a short time, transmission of diseases by these pathogens, may be eliminated by aging the seed beyond the time required for death of the pathogen concerned.

The effect of storage on the microflora of clover seed was studied using seed samples examined during the survey. Twelve samples were selected on the basis of the pathogenic fungi carried by the seed. Unfortunately all the pathogens encountered were not present in seed lots harvested during the same year. This made it necessary to divide the samples into two groups of six, dependent on whether they were harvested in 1953 or 1954. At the start of the storage period, 200 untreated seeds from each sample were plated on malt extract agar and the microflora determined. The seed was then stored at room temperature in 5 cc. corked vials. The microflora was redetermined after two years[12] for samples harvested in 1953, and after one year for those harvested in 1954. The results are shown in Table XI.

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[12]Two years was the maximum that samples could be stored in the time available for this study. However, the same samples are still under storage and the microfloras will be redetermined after three, four and five years.

Table XI. The effect of storage at room temperature on the longevity of seed-borne fungi.

Fungus	Seed stored for one year, 1954-1955.[1]		Seed stored for two years, 1953-1955.[1]	
	Initial population	Final population	Initial population	Final population
	Number	Number	Number	Number
Cladosporium cladosporides	549[2]	304[2]	845[2]	170[2]
C. elatum	18	36	-	1
Penicillium spp.	12	7	33	20
Phoma trifolii	1	20	17	10
Botrytis cinerea	16	3	13	1
Stemphylium botryosum	73	73	0	11
Stemphylium consortiale	66	14	24	4
Stemphylium sp. (Pseudoplea?)	19	2	-	-
Mucoraceous spp.	9	10	3	16
Aspergillus spp.	6	1	5	15
Verticillium sp.	8	2	-	-
Fusarium roseum	-	-	4	0
Ascochyta imperfecta	-	2	10	-
Alternaria tenuis	115	89	67	24
Epicoccum sp.	26	40	30	10
Sclerotinia sp.	-	-	54	1[2]
Pullularia sp.	-	1	1	-
Total	930	607	1096	286
Per Cent Reduction		35		74

[1]Seed stored for one year, was composed of different samples from that stored for two years.

[2]Total number of fungi observed from 6 samples of seed (200 seeds per sample plated).

The overall reduction in the micro-flora of clover seed after storage at room temperature for one year, was 35 per cent, and after two years was 74 per cent (Table XI). Certain species were significantly reduced after storage for two years whereas, others were apparently unaffected.

Several fungi, Verticillium and Pullularia for example, were present in such small numbers in the samples, that it was not possible to judge the effect of storage on them on the basis of the 200 seeds employed.

Stemphylium botryosum, Aspergillus spp. and mucoraceous fungi, were actually present in greater numbers at the termination of the two year storage period. The increase in counts was consistent among the infested samples and was therefore not considered to be due to the expression of variability among the samples. The reason for this increase is not known. A possible explanation is that at the initial count these fungi were overgrown or inhibited by other competing organisms, but after a period of storage, many of the competitors had lost their viability, or were weakened, allowing the overgrown species to reveal themselves.

It is worthy of note that with the exception of a single viable sclerotium which was mistakenly plated as seed, the Sclerotinia was completely eliminated after two years storage. It would seem that this Sclerotinia (Appendix A) is viable at room temperatures in a mycelial state for only relatively short periods. Ascochyta imperfecta was also eliminated within two years.

CHAPTER IX

THE RELATIVE EFFECTIVENESS OF CARBON TETRACHLORIDE, WATER AND AIR AT 158°F, AS ERADICANTS OF SEED-BORNE FUNGI

In Chapter III, Oregon grown clover seed was shown to carry many fungi some of which were potential pathogens. In the ensuing study an effort was made to eradicate these seed-borne fungi by means of heat treatment.

In spite of the fact that research on chemically treating clover seed has been proceeding for over a decade (3, 10, 58, 107, 109), heat treatment has received little attention. The lack of knowledge of the effect of heat on the fungus population of clover seed motivated the following investigation on the relative effectiveness of carbon tetrachloride, water and air at 158°F as fungus eradicants of clover seed.

Thermal treatment of seed for many years has consisted of heating seed in water or less often in air, at temperatures ranging between 100°F and 130°F. Water as a medium for transferring heat to seed has important disadvantages in that the seeds must either be sown or dried soon after immersion, and the seed is easily injured. Recently carbon tetrachloride was used experimentally as a medium for heating seed (70). This chemical has two distinct advantages over water, its high volatility eliminates the necessity of drying the seed after immersion, and it can be used at much higher temperatures than water, without injury to the seed. Disadvantages are found in its higher cost and its toxicity to humans.

Seed of crimson clover infested with Cladosporium cladosporioides, Alternaria tenuis, Fusarium roseum, Epicoccum sp., Phoma trifolii, Stemphylium botryosum, S. consortiale, Penicillium sp. and a Sclerotinia sp., were thoroughly mixed with a "Gamet" divider to obtain uniformity. The sample was then divided into 32, 3 gm. lots, each of which was tied in a small cheese cloth sack. The sacks were divided into three lots and placed independently in water, carbon tetrachloride and air heated to 158°F.[13] A single sack of seeds was removed from each of the media after 1, 4, 8, 12, 24, 36, 48, 60, 72 and 84 hours immersion. Tests were then made on the amount of fungus infestation and germinability of the seeds.

Fungus infestation was determined by plating 160 treated seeds on malt agar and incubating at 21°C (seed heated in water was dried in partially open petri dishes to enable plating with a vacuum counter). Germination of treated seed was determined by repeating the above procedure using moist filter papers instead of malt agar.

In addition to the above treatments, one sack of seed was immersed in carbon tetrachloride at room temperature to determine whether or not the carbon tetrachloride was fungicidal at ordinary temperatures.

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[13] 500 ml. of water and carbon tetrachloride in stoppered Erlenmeyer flasks held at 158°F (± 0.5) in a thermostatically controlled hot water bath. A constant temperature oven was used for the air treatment.

Table XII. The effect of heat (158°F) on seed germination and fungus microflora of crimson clover seed.

Medium	Length of treatment	Micro-organisms: Alternaria	Cladosporium	Epicoccum	Fusarium	Penicillium	Phoma	Sclerotinia	Stemphylium botryosum	Stemphylium consortiale	Infestation[1]	Germination[2]
	hours	Numbers[3]									Percent	Percent
	1	-	2	-	-	1	-	-	-	-	5	-
	4	-	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-	-
Water	12	-	-	-	-	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-	-	-	-	-
	36	-	-	-	-	-	-	-	-	-	-	-
	48	-	-	-	-	-	-	-	-	-	-	-
	60	-	-	-	-	-	-	-	-	-	-	-
	72	-	-	-	-	-	-	-	-	-	-	-
	84	-	-	-	-	-	-	-	-	-	-	-
	1	24	26	4	-	-	2	-	-	4	51	88
	4	3	9	-	-	-	1	-	-	3	13	58
	8	1	-	-	-	-	-	-	-	-	-	26
Air	12	-	-	-	-	-	-	-	-	-	-	57
	24	-	-	-	-	-	-	-	-	-	-	20
	36	-	-	-	-	-	-	-	-	-	-	-
	48	-	-	-	-	-	-	-	-	-	-	-
	60	-	-	-	-	-	-	-	-	-	-	-
	72	-	-	-	-	-	-	-	-	-	-	-
	84	-	-	-	-	-	-	-	-	-	-	-
	1	7	26	1	2	-	-	-	1	2	34	98
	4	2	25	1	1	-	1	-	2	1	28	98
	8	5	29	3	-	-	-	-	-	2	34	98
C Cl_4	12	6	26	-	-	-	-	4	1	1	34	88
	24	4	21	-	-	2	-	1	-	-	24	89
	36	3	22	-	-	1	-	2	-	1	25	93
	48	2	21	1	-	-	-	1	-	1	22	89
	60	1	17	-	-	2	-	-	-	2	19	88
	72	-	4	-	-	1	-	1	-	4	10	90
	84	3	5	-	-	-	-	-	-	-	7	95
C Cl_4 (Room temp.)	84	18	72	8	4	-	11	-	-	2	100	88
Untreated seed	24	-	55	7	3	3	15	-	3	4	100	100

1 Percentage infestation relative to untreated seed.
2 Percentage germination relative to untreated seed.
3 Number of times each fungus occurred on 160 seeds plated on malt agar.

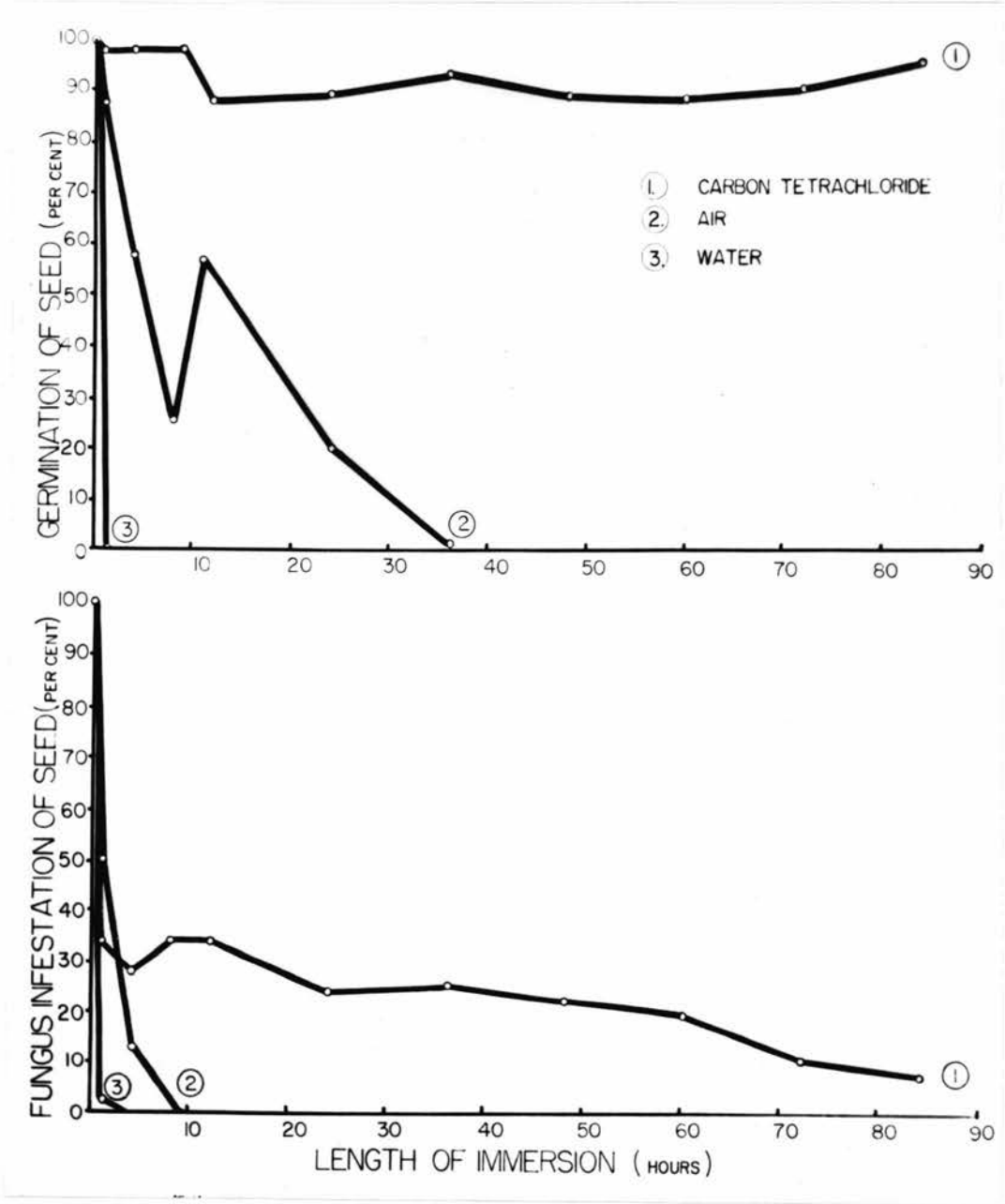

Figure 16. Effect of temperature (158°F) on the microflora and seed germination of crimson clover seed heated in water, carbon tetrachloride and air.

Results

Temperatures of 158°F in all three media caused marked reduction of the microflora following the minimum immersion of one hour (Table XII and Figure 16). Water destroyed 99 per cent of the microflora and carbon tetrachloride and air destroyed 66 and 49 per cent respectively. The microflora of oven heated seed was completely eradicated after 8 hours at 158°F. Seed in carbon tetrachloride showed only a slow rate of decrease of infestation after the initial rapid reduction, with a 93 per cent destruction reached only after 84 hours (Figure 17). Complete eradication in carbon tetrachloride was not accomplished within the time of the experiment.

Heat treatment of seeds in water, carbon tetrachloride and air differed significantly in their effect on germination (Table XII and Figure 16). All seeds were killed after one hour in water at 158°F whereas carbon tetrachloride failed significantly to effect the germinability of immersed seed even after the maximum steep of 84 hours. Oven heated seeds showed a marked reduction of germination after the one hour treatment but complete death of the seed did not occur until after 36 hours.

Table XII shows the effect of heating in the three media, on the individual fungus species present on the seed. Cladosporium and Penicillium were the only fungi able to survive one hour at 158°F in water. Neither of these survived in water for longer periods of immersion. Alternaria, Cladosporium, Epicoccum, Phoma, and Stemphylium consortiale, were able to survive 158°F air for one hour, although there was a considerable reduction of all species except

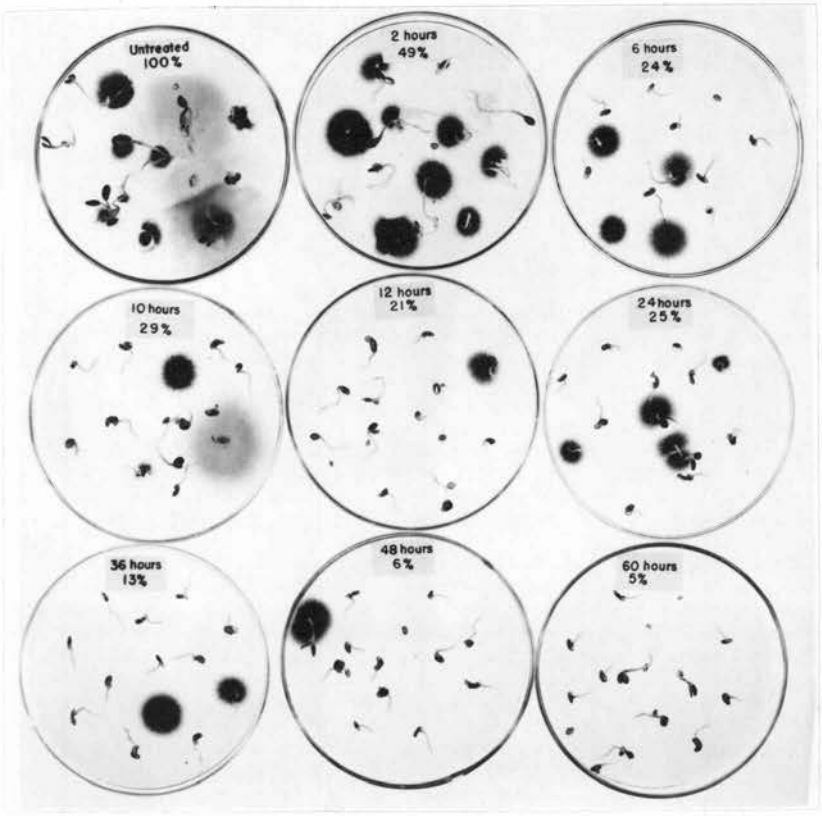

Figure 17. Reduction of the microflora of crimson clover seed resulting from the immersion of seed in carbon tetrachloride at 158°F. (Percentages represent the amount of infestation compared to untreated seed). (Photo. by H. H. Millsap)

Alternaria and Stemphylium botryosum. No fungus was able to survive eight hours in air at 158°F. In carbon tetrachloride, the potentially pathogenic species, Fusarium roseum, Phoma trifolii and Stemphylium botryosum were eradicated from the seed after four, four and twelve hours respectively, though the Sclerotinia sp. (present with the seed in a mycelial state) was still viable after 72 hours. The saprophytic fungi were greatly reduced with carbon tetrachloride as the heating medium, though a few were viable even after a steep of 84 hours (Figure 17).

Seed steeped for 84 hours in carbon tetrachloride kept at room temperature, showed no significant decrease of fungus infestation when compared with the untreated seed. The carbon tetrachloride apparently had no fungicidal effect on the seed microflora at room temperature. Whether this was also true at higher temperatures was not determined.

The ability of the seed to withstand high temperatures in carbon tetrachloride without injury, is believed to result from exclusion of oxygen and retardation of oxidation processes within the seed.

CHAPTER X

SUMMARY AND CONCLUSIONS

Seed of Oregon grown clovers - red, crimson, alsike and ladino, were found to carry the following fungi: Alternaria tenuis, Ascochyta imperfecta, Aspergillus fumigatus, A. niger, A. ochraceous, Botrytis cinerea, Cephalosporium sp., Cephalothecium sp., Chaetomium cochliodes, C. funicola, Cladosporium cladosporioides, C. elatum, C. macrocarpum, Didymium difforme, Epicoccum sp., Fusarium roseum, F. oxysporum, mucoraceous spp., Nigrospora oryzae, Penicillium javanicum, P. novae-zeelandiae, Penicillium spp., Phoma trifolii, Pullularia sp., Rhizopus sp., Sclerotinia sp., Scopulariopsis brevicaulis, Stemphylium consortiale, S. botryosum, S. sarcinaeforme, Stemphylium sp., (Pseudoplea?), Trichoderma sp., Verticillium, a yellow bacterium and a white bacterium. Of this group only Alternaria tenuis, Ascochyta imperfecta, Botrytis cinerea, Chaetomium cochliodes, Fusarium roseum, Nigrospora oryzae, Penicillium spp., Sclerotinia sp., Stemphylium botryosum and S. sarcinaeforme are reported in the literature as associated with clover seed.

Among the fungi listed, are a number having previous histories of parasitism. These are: Ascochyta imperfecta, Botrytis cinerea, Fusarium roseum, Phoma trifolii, Sclerotinia sp., Stemphylium botryosum, S. sarcinaeforme, Stemphylium sp. (Pseudoplea?) and Verticillium sp. (or spp.?). From six to 47 per cent of all the samples examined were carrying one or more of these pathogens. Greatly outnumbering the potentially pathogenic forms, were those

generally referred to in the literature as saprophytes or at most weak parasites.

The survey results, failed to bear out the expectations held at the beginning of the study, that significant qualitative and quantitative differences would be found among seed microfloras from different climatic and geographic regions of Oregon, as well as between different species of clovers. Qualitatively there was little difference between the regions, though quantitatively moulds were as a rule, more abundant on Willamette Valley seed than on seed produced in the drier regions. The composition of the microfloras of red, alsike, crimson and ladino clovers was similar. With few exceptions, most of the fungi were common to all four species.

The conclusion drawn from the survey, was that the seed of Oregon grown clovers which is exported to many parts of the United States, as well as to foreign countries, is transmitting in a viable state, a number of fungi which are potentially serious pathogens of clover.

Pathogenicity tests with fungi isolated from clover seed demonstrated that a number of these organisms when artifically inoculated onto seeds in soil tests, were capable of greatly reducing the emergence of seedlings. Ascochyta imperfecta caused damping-off and necroses of 79 per cent of the seedlings; Botrytis cinerea, 95 per cent; Fusarium roseum, 62 per cent; Stemphylium botryosum, 47 per cent; and Phoma trifolii, 85 per cent. Isolates of Cladosporium cladosporioides, Stemphylium sp. (Pseudoplea?), Sclerotinia sp., and Alternaria tenuis showed weak pathogenicity

in seedling tests. In tests on mature clover plants, a species of Verticillium was the only fungus of those tested, that was consistently reisolated from inoculated roots. The pathological significance of the transmission of this Verticillium with clover seed could not be evaluated from the data available.

Though severe pathogenesis of seedlings by fungi isolated from Oregon clover seed is established, the more important question of the significance of seed transmission in field sown seed, is unanswered; for this is a far more complex problem than could be solved in the time allotted for this study.

Cladosporium cladosporioides, Cladosporium elatum, Verticillium sp., Aspergillus spp., Stemphylium consortiale, Rhizopus sp., Trichoderma sp., Botrytis cinerea, Fusarium roseum, Ascochyta imperfecta and Scopulariopsis brevicaulis were found to be carried loosely on the seed presumably as spores or mycelium. Practically every organism encountered during the survey was able to penetrate to some degree into the seed coat or deeper, and thus escape complete elimination by surface disinfection. The dark hyphae of Cladosporium cladosporioides, ramifying over the seed coat were often visible at low magnifications. Certain fungi were able to exist as sclerotia[14] mixed with the seed, and an unidentified Stemphylium (Pseudoplea?),

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[14]The few sclerotia plated for identification, proved to be species of Sclerotinia. It would be presumptious to assume that all the sclerotia observed belonged to this genus. (See Chapter II under "Sclerotial forms").

was found carried on the seed coat as minute sclerotia.[15]

Conclusions drawn from this study were that most of the species of fungi encountered, were able to survive in a microscopic form both loosely on the seed coat and deeper within the seed. Only a few of the species occurred in a form that was visually detectable on the dry seed.

Analysis of the air over a field of crimson clover yielded no fungi that had not been observed previously on clover seed during the survey work. Eighty-two per cent of the fungi present on the seed harvested from this field were also present in the air. Many of the fungi encountered on clover seed during the survey are species commonly present in the air.

The respiration of the microfloraof germinating alsike clover seed was studied in a Warburg respirometer. Measurement of the oxygen uptake indicated that the respiration of the microflora constituted a significant part of the total respiration of the untreated, germinating seeds.

The longevity of organisms occurring on naturally infested clover seed stored at room temperature was studied over a period of two years. One group of samples stored for one year, showed a 35 per cent reduction of fungi whereas another group after storage for two years showed a 74 per cent reduction. Cladosporium cladosporioides, Penicillium spp., Phoma trifolii, Botrytis cinerea, Stemphylium

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[15]Based on circumstantial evidence.

consortiale, Stemphylium sp. (Pseudoplea?), Fusarium roseum, Ascochyta imperfecta, Alternaria tenuis and a Sclerotinia sp. were either reduced or eliminated after two years, whereas Stemphylium botryosum, Cladosporium elatum, a mucoraceous fungus, and Aspergillus spp. were apparently unaffected. It is concluded that, although storage of clover seed at room temperature will eliminate or reduce certain fungus pathogens in clover seed, the practicality of such a method for obtaining healthy seed is doubtful.

The comparative effect of heat of 158°F on seed germination and fungus survival was studied employing water, air and carbon tetrachloride as ambients. At 158°F, immersion of seed in both air and water caused complete eradication of fungi after one and eight hours respectively. However, seed germination was seriously reduced after the minimum treatment of one hour. Carbon tetrachloride had no adverse effect on germination even after the maximum treatment of 84 hours, although the seed-borne fungi were greatly reduced, with the potential pathogens most severely damaged. Fusarium roseum, Stemphylium botryosum and Phoma trifolii were eradicated after 4, 4 and 12 hours respectively.

Carbon tetrachloride at 158°F when used as an ambient for crimson clover seed, significantly reduces the fungus microflora (particularly certain pathogenic species), without impairing the germinability of the seed. Water and air at 158°F are considered unsuitable because of the severity of injury to the seed.

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APPENDIXES

APPENDIX A

Included in Appendix A are notes on fungus species isolated from clover seeds that were difficult to identify, or whose identities were considered to be in need of further clarification from a taxonomic viewpoint.

Epicoccum sp. - was sterile at room temperature and therefore not easily identifiable by one unfamiliar with this fungus. It grew quite rapidly on malt agar at 21°C and produced colonies that were initially white but which soon became tinged with a reddish-pink color. The young colonies closely resembled certain species of Fusarium. The older cultures were colored shades of yellow or orange. Small, regular, black sclerotia formed in the young colonies. These sclerotia became knobbly and gnarled as the cultures aged. Only one isolate was observed to produce conidia at room temperatures. Usually conidia were produced between 5 and 10°C, particularly on small pieces of colonies partially submersed in sterile water.

The Epicoccum encountered during this study has similarities with the unidentified fungi noted by Evans (27) and Fergus (28).

Fusarium spp. - Isolates of F. roseum[16] exhibited a confusing array of spore and colony types which is normal for this species. The single culture of F. oxysporum observed was quite typical for this

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[16] Confirmed by Dr. W. G. Snyder of the University of California at Berkeley.

species.

Sclerotinia sp. - was present as mycelia on and within crimson clover seeds from the Hillsborough area. Culturally it appeared to be either S. trifoliorum or S. sclerotiorum, though according to Noble,[17] neither of these fungi has been proven to be transmitted as mycelia with clover seeds. Species identification was not possible because the fungus could not be induced to produce apothecia. This inability to produce apothecia, may have resulted from prolonged storage on malt agar slopes. Micro-conidia (spermatia) were produced abundantly in culture (Figure 31). Keay (53 p. 242) states that S. trifoliorum "...produces small waxy globules of micro-conidia; these waxy bodies have never been observed in cultures of S. sclerotiorum." If this is a valid criterion for the separation of the two fungi, then the isolates from crimson clover were S. trifoliorum. However, Purdy (87 pp. 421-427) suggests combining these and other species under the one species[18] S. sclerotiorum. General adoption of such a combination, would place the crimson clover isolates in the species S. sclerotiorum var. "major".

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[17] Personal communication from Dr. Mary Noble (Seed Testing, Plant Registration, and Plant Pathology Station, Edinburgh, Scotland.) dated May 3, 1954.

[18] In a letter dated March 3, 1956, Dr. Noble states in regard to Purdy's suggestion of combining S. trifoliorum and S. sclerotiorum "... it confirms what I have been thinking for some time that there is indeed little difference between the two so called species."

Stemphylium sp. (Pseudoplea?) - The identity of this fungus is still to be determined. At room temperature it produced a dark colony in which were embedded a great many small sclerotia (or incipient perithecia?) (Figure 36). Colonies on malt agar were very similar to published photographs and descriptions of Pseudoplea trifolii (Chapter II), though the fungus could not be induced to produce fertile perithecia by the procedures recommended for P. trifolii.

When pieces of colony fragments were partially submersed in sterile water and incubated at temperatures ranging from 5° to 30°C, conidia of the genus Stemphylium, were produced sparsely at 5, 10 and 15°C. These conidia (Figure 35) were not typical of any species described on clover. To establish that the conidia were not produced by a contaminant, single spore isolations were made. The single Stemphylium spores produced "Pseudoplea" like colonies.

Cultures were sent to Dr. L. E. Wehmeyer[19] who stated that the colonies were typical of the genus Pseudoplea. Dr. Wehmeyer was dubious about the formation of a Stemphylium stage by Pseudoplea, but stated that if such did occur, he would have to revise some of his ideas. Dr. Emory G. Simmons also examined cultures[20], and stated that the Stemphylium stage of this fungus was different to any of the species of this genus that he had seen. Dr. J. H. Graham, informed

[19]Personal communication, October 21, 1955.
[20]Personal communication, January 30, 1956.

of the writer's work through Dr. Simmons,[21] stated that he had been working with a similar fungus, but had isolated it only from ladino clover leaves where it had caused irregular, light brown necrotic areas up to 5 mm. diameter. These lesions were usually marginal.

It seems probable that this Stemphylium is an undescribed[22] species. Whether it possesses a Pseudoplea perfect stage is still to be determined.

Verticillium sp. (or spp.?) - were not typical of either V. albo-atrum or V. dahliae. Isolate 70NS which showed pathogenicity on the mature plants produced dense, snow white colonies on malt agar (Figure 41), and like all other isolates, failed to produce chlamydospores or sclerotia on malt agar, potato dextrose agar or sterilized tomato stems. It fruited profusely on malt agar at 21°C with the typical verticilliate sporophores (Figure 37). Isolate 101NS produced colonies that were dark brown to black when mature. Only a few of the sporophores of this isolate were verticilliate. The majority were more typical of the genus Cephalosporium. In culture, isolate 101NS exuded a strong, sweet, mealy odor.

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[21] Personal communication, February 8, 1956.

[22] A rough draft of a manuscript by Dr. J. H. Graham entitled "A Stemphylium Disease of Ladino Clover" was received by the writer after the final typing of this thesis. In this manuscript, the Stemphylium independently observed by Dr. Graham and the writer, is described as Stemphylium repeni sp. nov.

Identification of the Verticillium was attempted. Descriptions by Rudolf (91 pp. 244-254), and Isaac (42; 43; 44; 45) did not satisfactorily describe the species from clover seed.

APPENDIX B

Figure XIII. The results of greenhouse tests in which both chemically treated and untreated seeds, heavily infested with Cladosporium cladosporioides were sown in sterilized and unsterilized soil.

	Seed Sown In Unsterilized Soil[1]			Seed Sown In Sterilized Soil
Sample	"Arasan" 2 oz. per 100 lbs.	"Arasan" 4 oz. per 100 lbs.	Untreated	Untreated
	Number	Number	Number	Number
Bur. 2687	78[2]	82	77	79
	80	77	78	76
	82	76	74	74
	(80)[3]	(78)	(77)	(77)
Bur. 2695	78	70	61	77
	69	75	71	64
	69	66	76	62
	(72)	(70)	(69)	(67)
Bur. 2473	59	54	66	69
	67	69	66	70
	59	70	62	60
	(62)	(64)	(64)	(63)
Bur. 2050	76	75	78	68
	70	73	70	78
	68	81	77	64
	(71)	(76)	(75)	(70)
Bur. 2059	67	70	70	66
	68	71	51	67
	62	64	71	60
	(66)	(68)	(64)	(64)

1 Seeds were sown in 3 replications of 100 seeds per rep.
2 Number of healthy seedlings harvested 21 days after sowing.
3 Mean of 3 replications.

APPENDIX C

Appendix C contains illustrations of certain fungi encountered with Oregon grown clover seed.

Microscopic drawings and photographs were made from water mounts of fungi grown on 2 per cent malt extract agar at 21°C. All drawings were freehand.

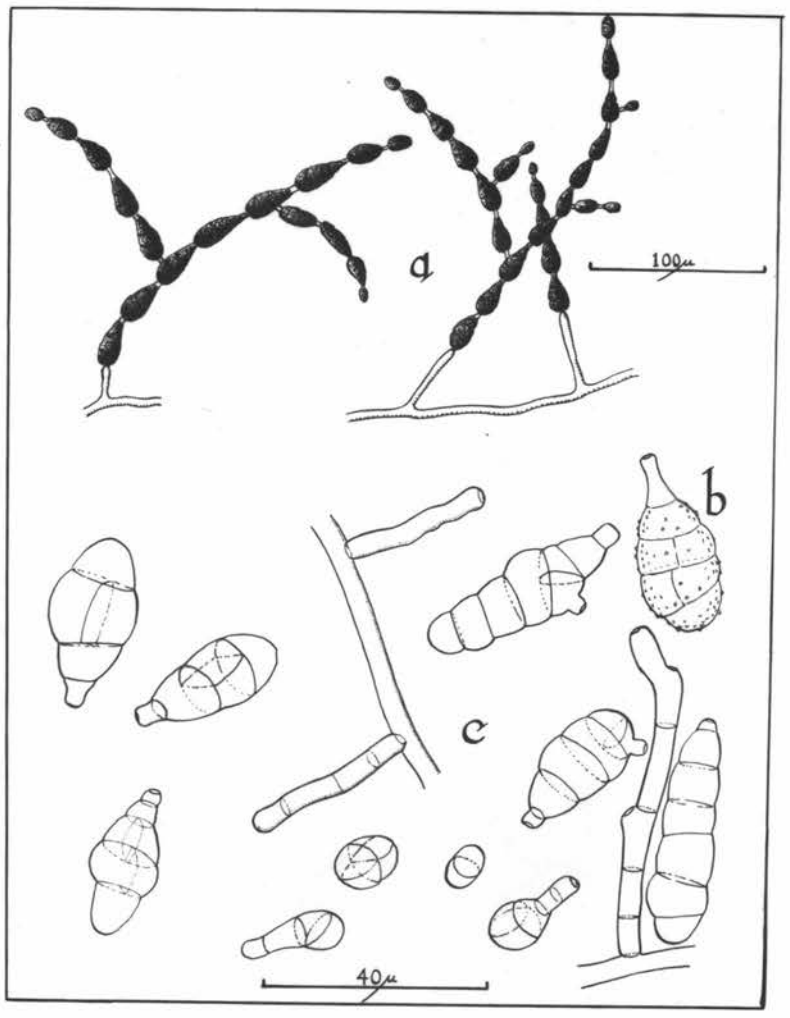

Figure 18. Alternaria tenuis auct. sensu. Wiltshire.
Upper) Chains of dark conidia borne on conidiophores.
Lower) Conidia and conidiophores.

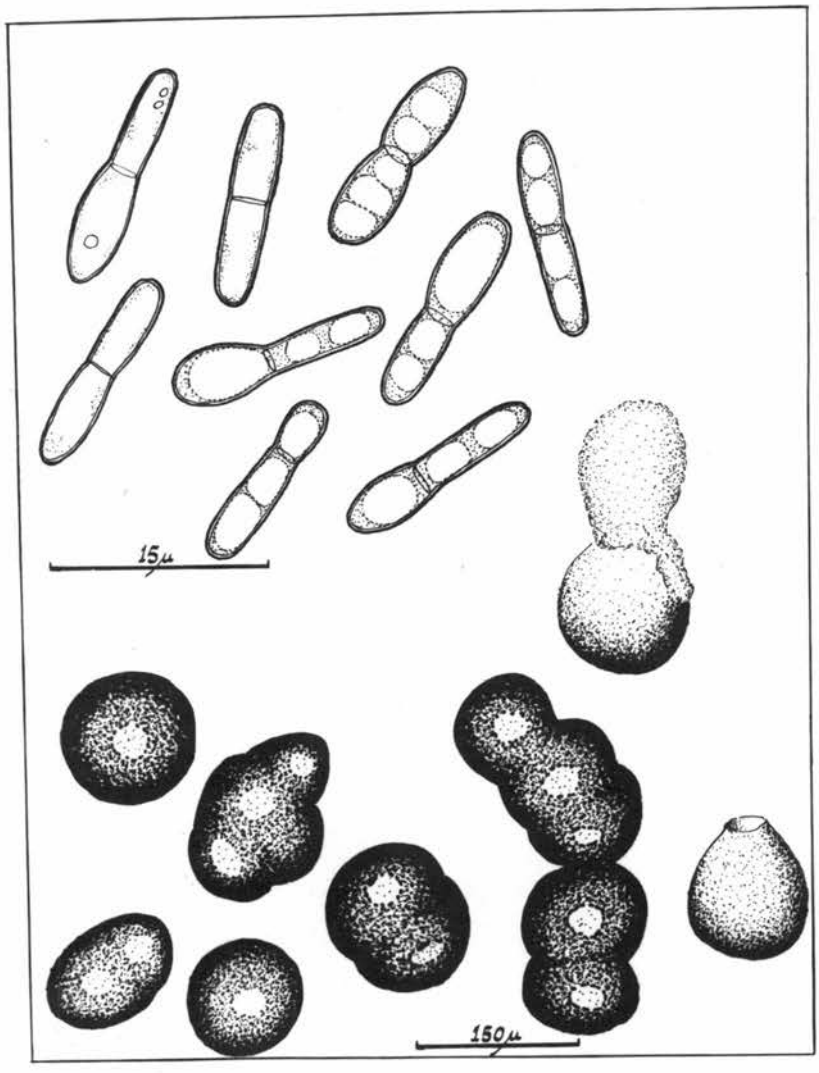

Figure 19. Ascochyta imperfecta Peck. Dark brown pycnidia and hyaline pycnidiospores.

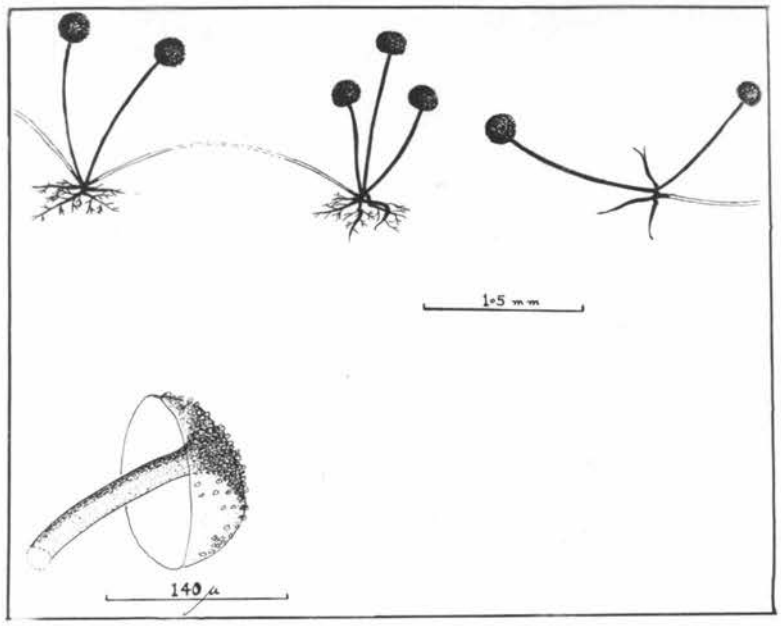

Figure 20. Rhizopus sp. Upper) Dark sporangiophores and sporangia. Lower left) Dehised sporangium.

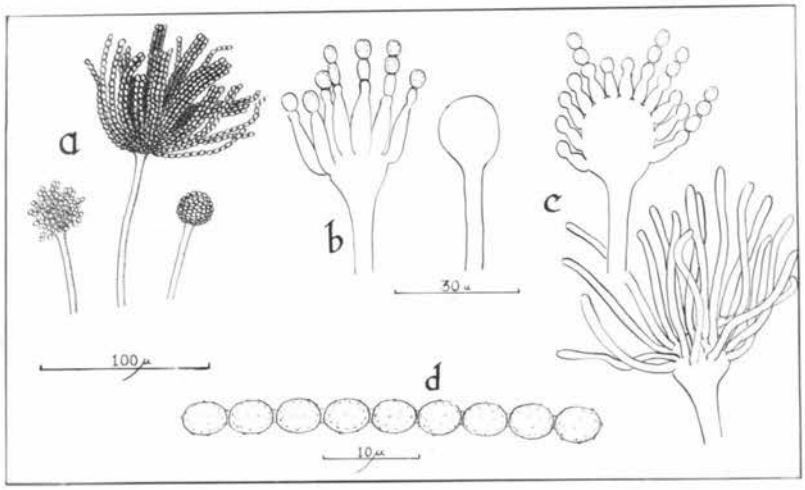

Figure 21. Aspergillus fumigatus Fresenius. a) Various stages of development of conidial heads. b) Sectional drawing of an atypical conidial head (left) and a young sporangiophore. c) Sectional drawing of a typical conidial head (upper), and a conidial head in which sterile stergmata have extended into hyphae (lower).

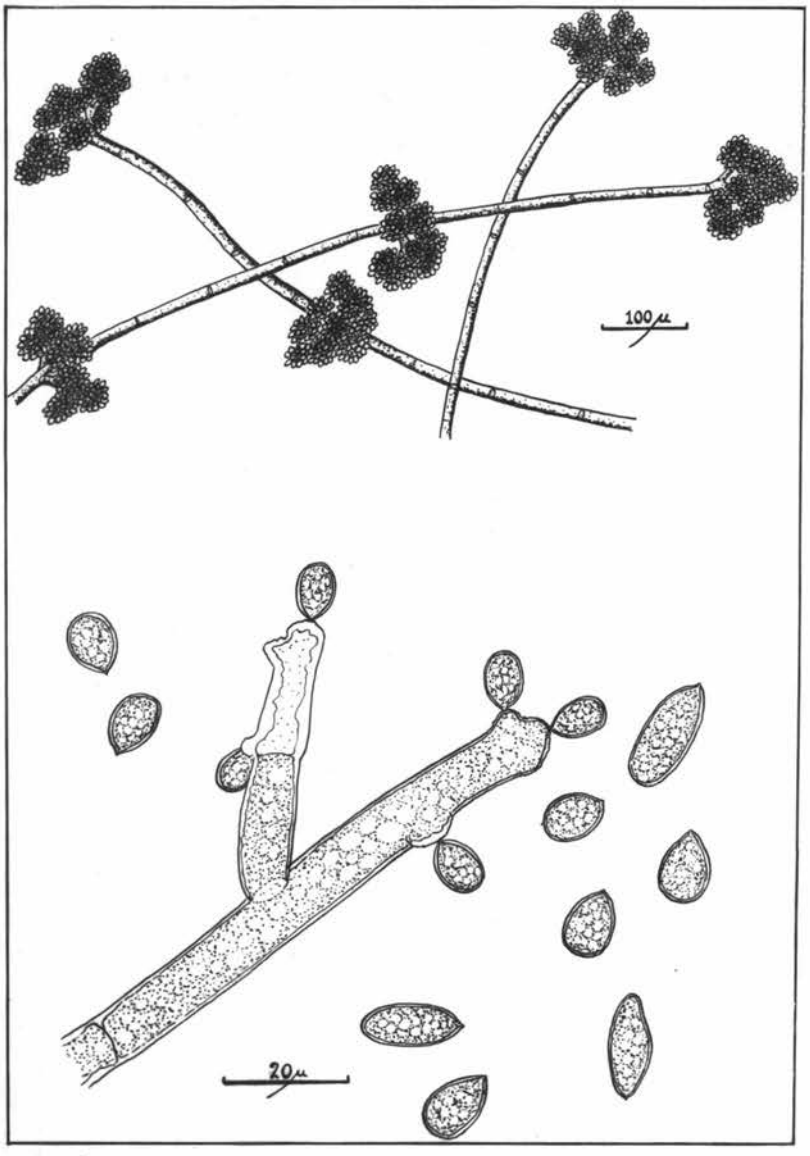

Figure 22. Botrytis cinerea Persoon. Upper) Dark colored conidiophores with grape-like bunches of conidia. Lower) Apex of conidiophore with attached and detached conidia.

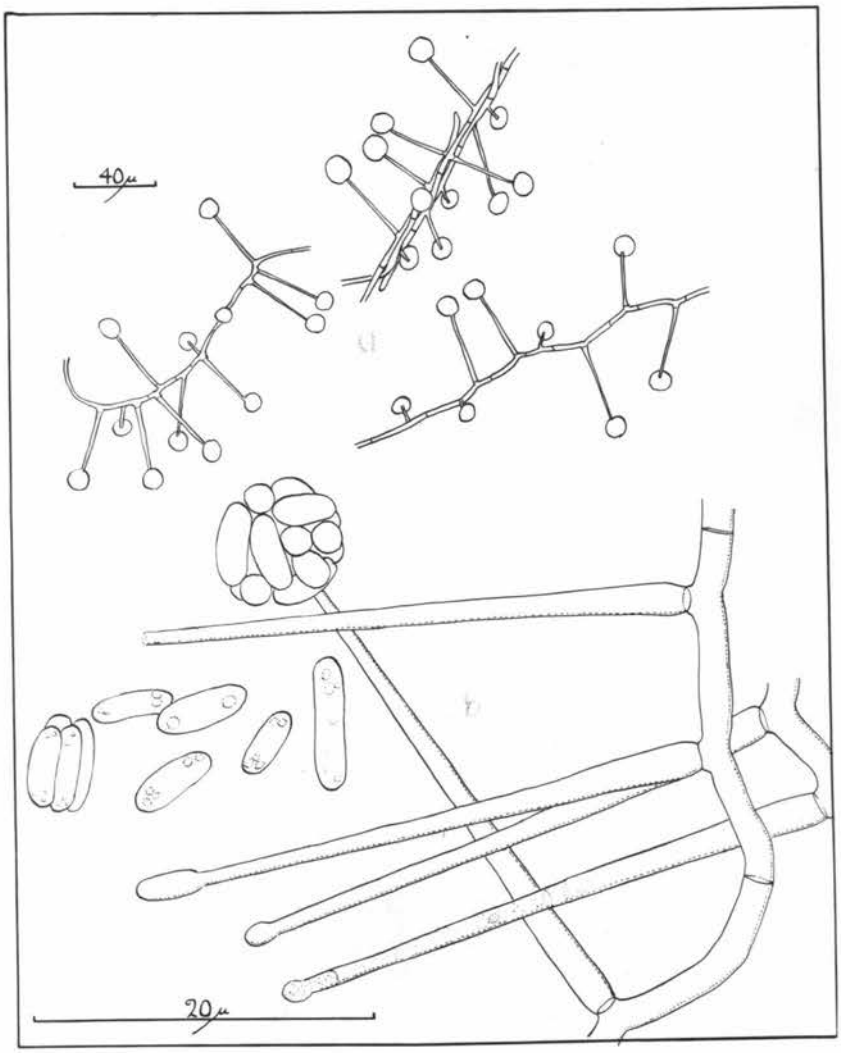

Figure 23. Cephalosporium sp. Upper) Conidiophores and globules of conidia. Lower) Hyaline conidiophores and conidia.

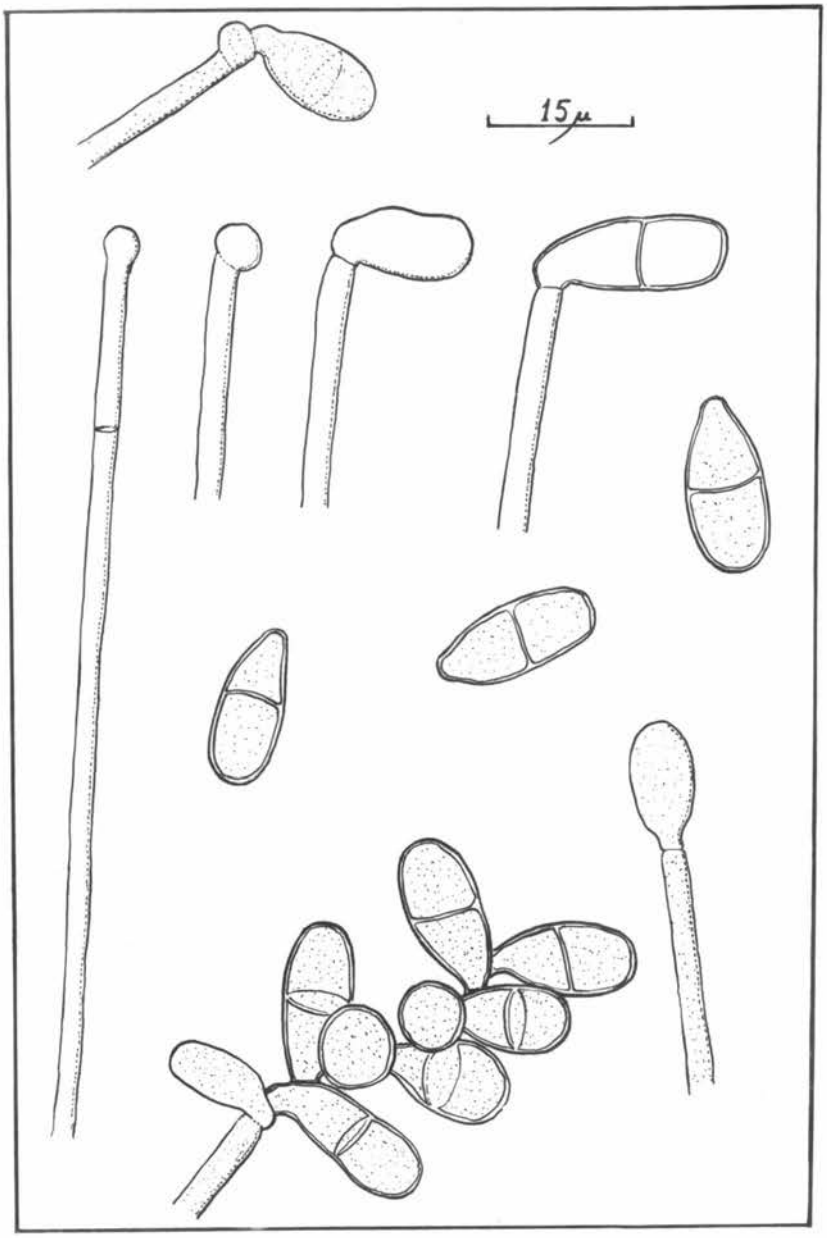

Figure 24. Cephalothecium sp. Upper) Various stages in the development of a conidium. Center) Mature hyaline conidia. Lower center) Conidiophore with conidia.

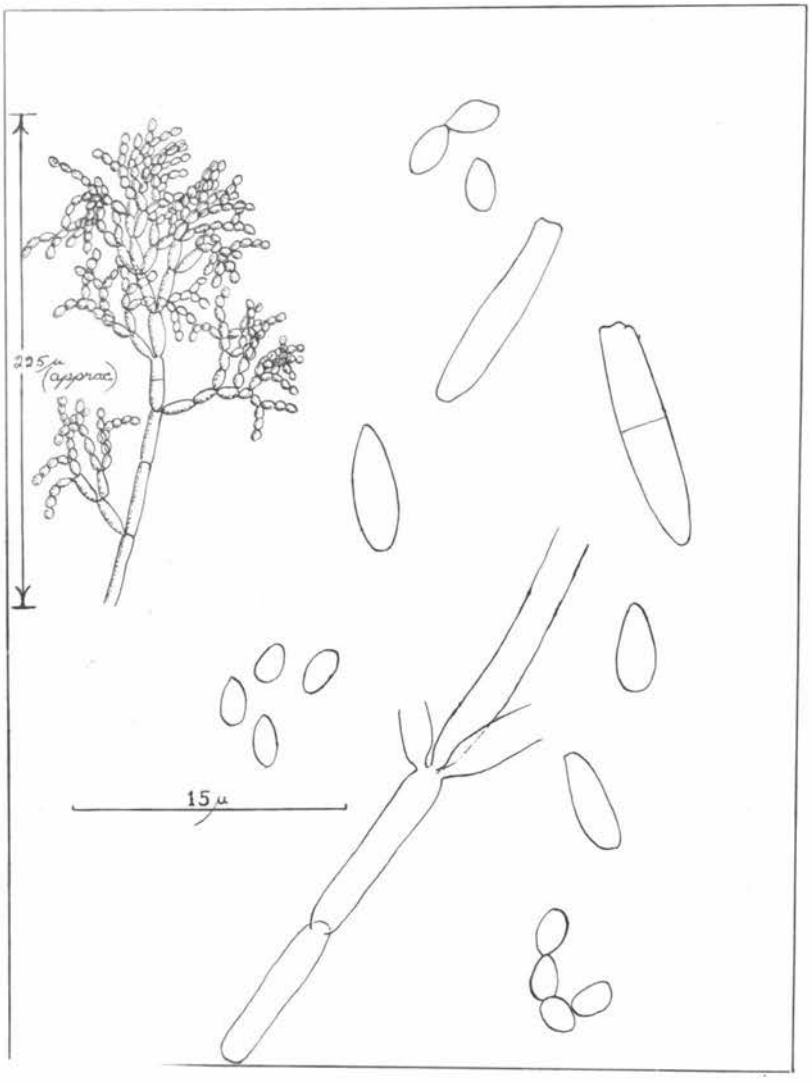

Figure 25. Cladosporium cladosporioides (Fres.) de Vries. Upper left) Conidial head. Remainder) Olivaceous brown conidia and portion of branching conidiophore.

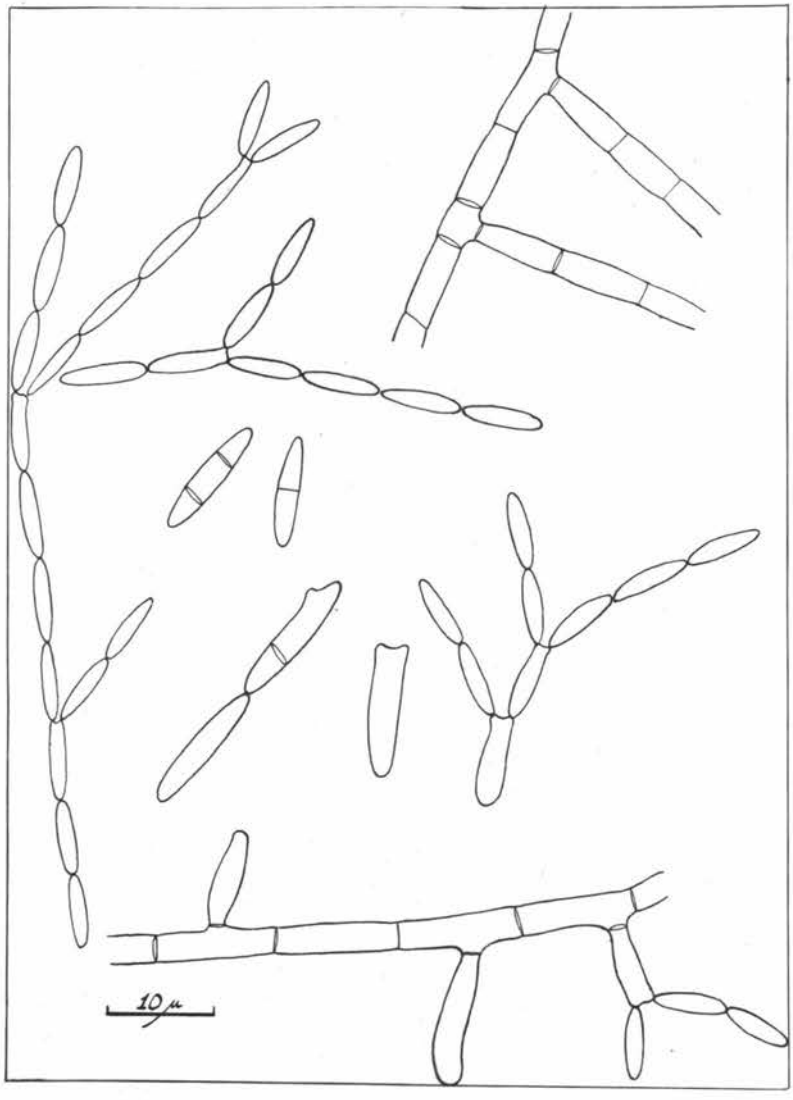

Figure 26. Cladosporium elatum (Harz) Nannfeldt. Yellowish brown conidia, conidiophores and hyphae.

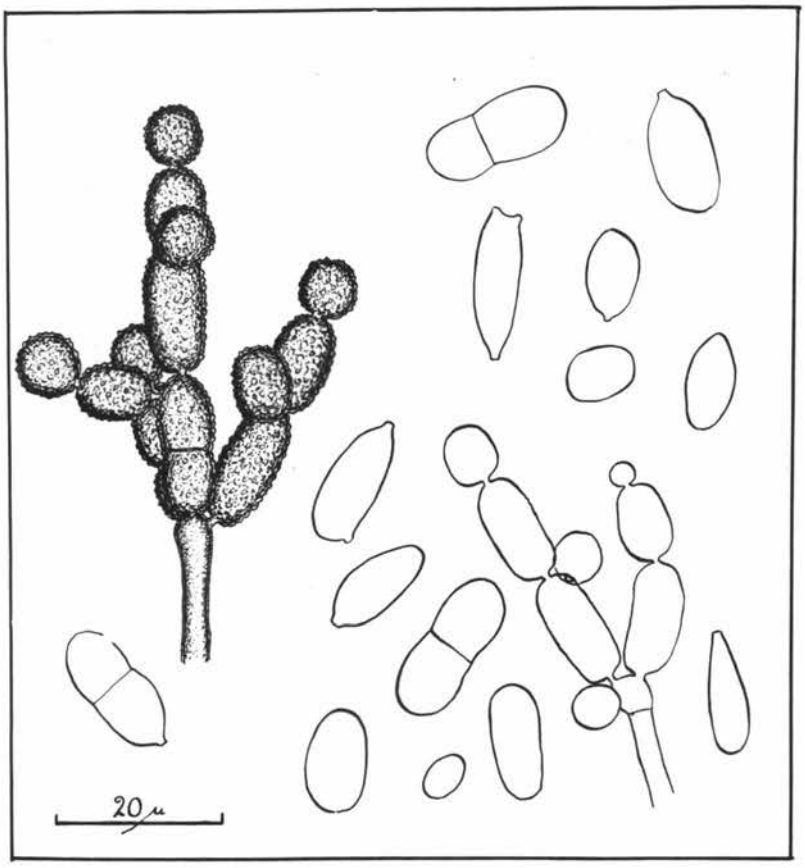

Figure 27. Cladosporium macrocarpum Preuss.
Upper left) Densely verrucose, olivaceous brown conidial head. Remainder) Outlines of conidia and conidiophore.

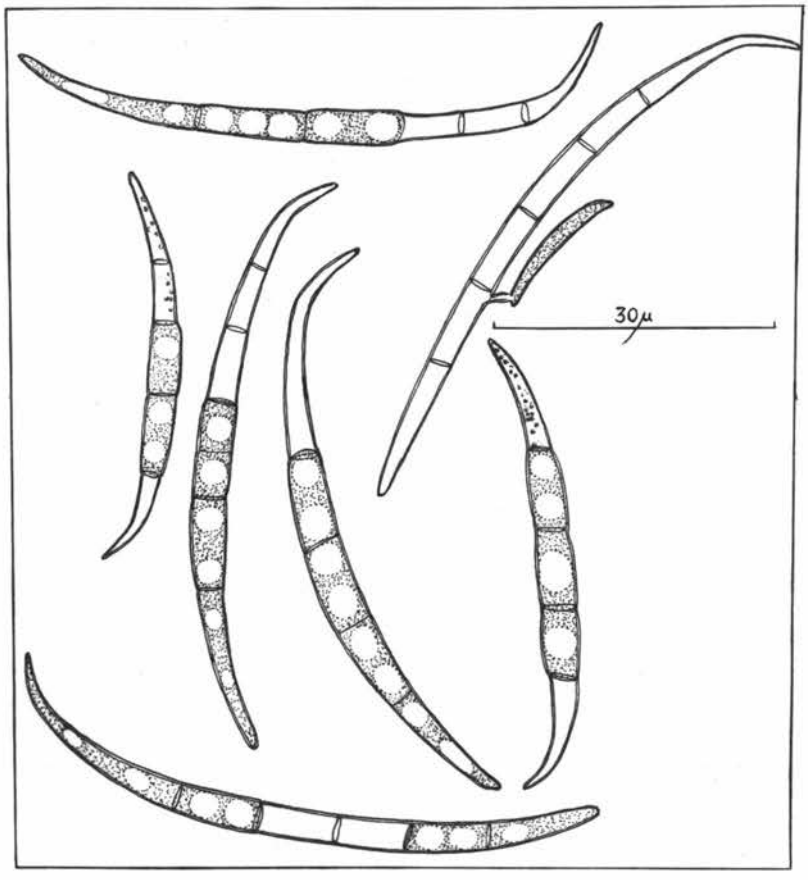

Figure 28. Fusarium roseum (Lk.) Snyder and Hansen. Hyaline macroconidia. (The macroconidia of different strains of F. roseum have a fairly wide diversity of forms. Portrayed above is the form most commonly encountered on clover seed.)

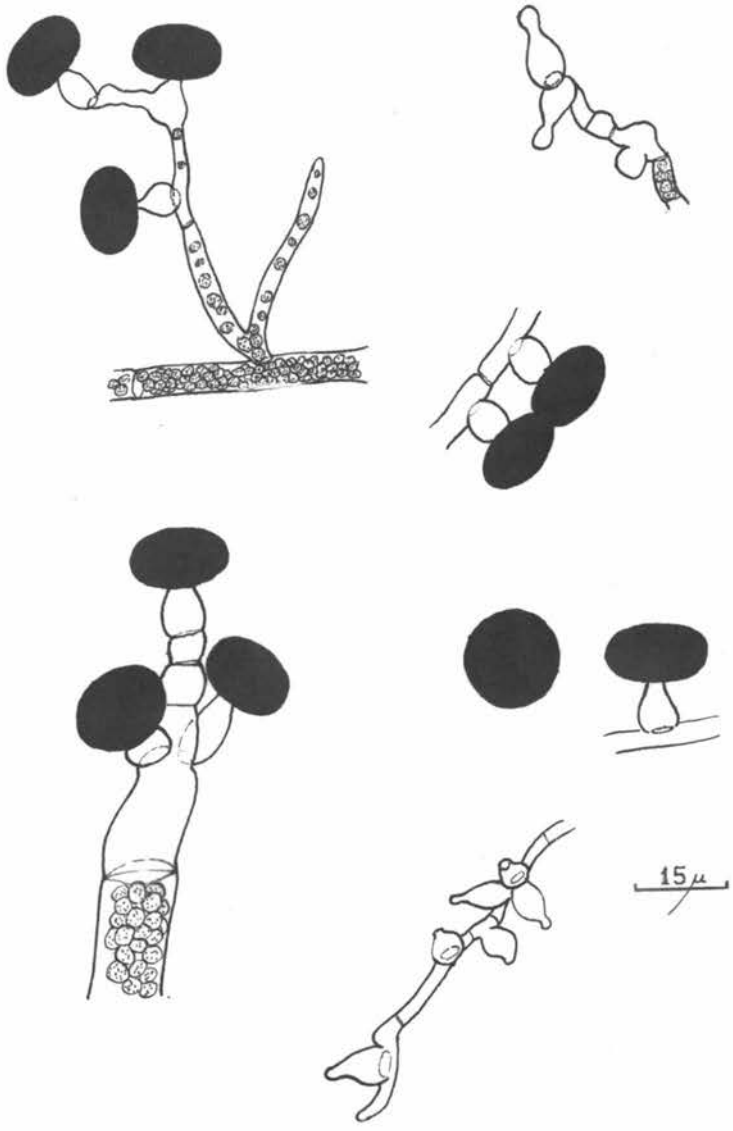

Figure 29. Nigrospora oryzae (Berk. and Br.) Petch. Hyaline conidiopores and black conidia.

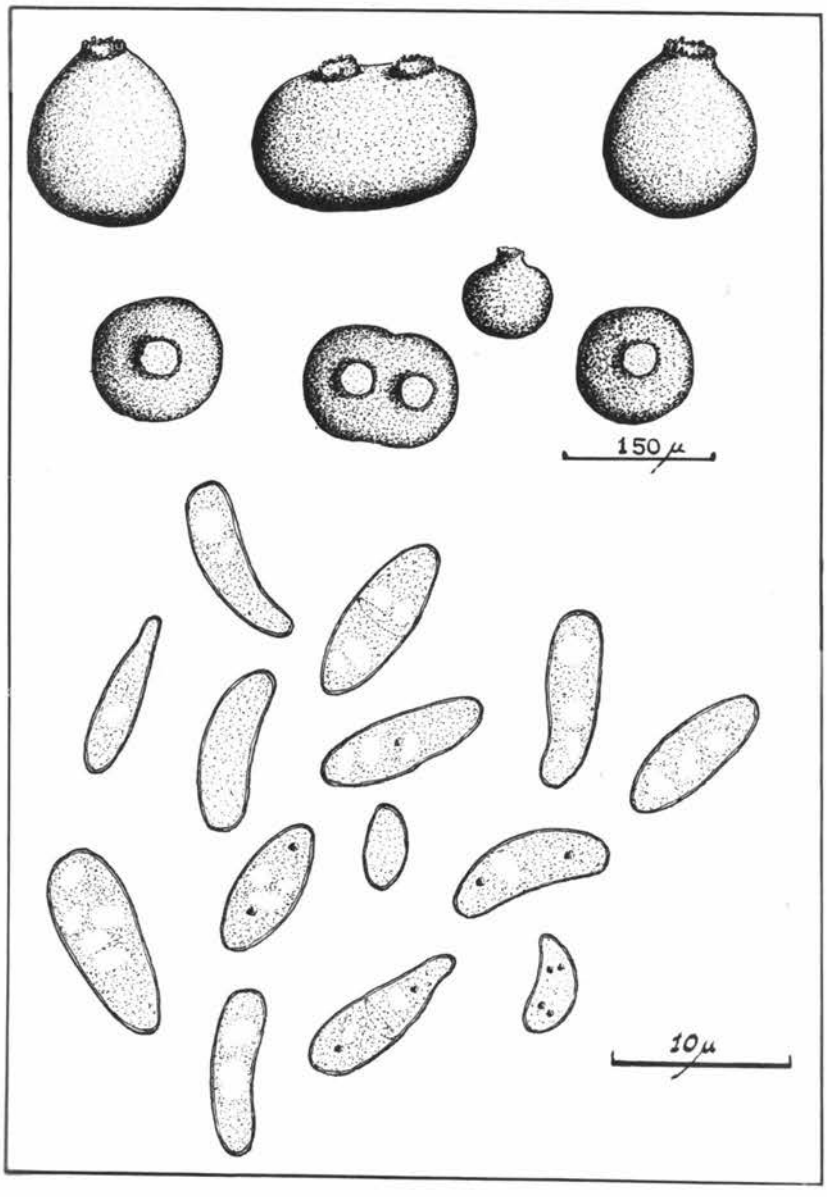

Figure 30. Phoma trifolii Johnson and Valleau. Brown pycnidia and hyaline pycnidiospores.

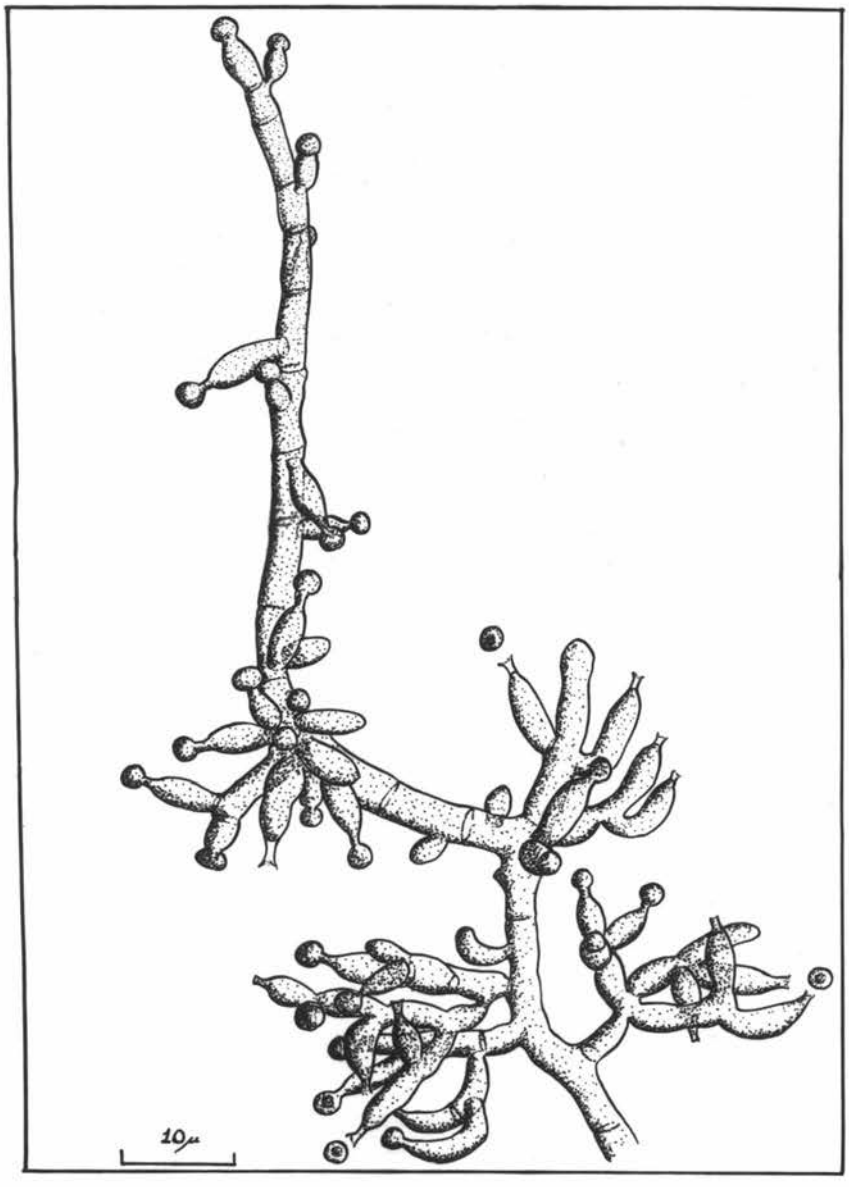

Figure 31. Sclerotinia sp. Spermadochium with spermatia.

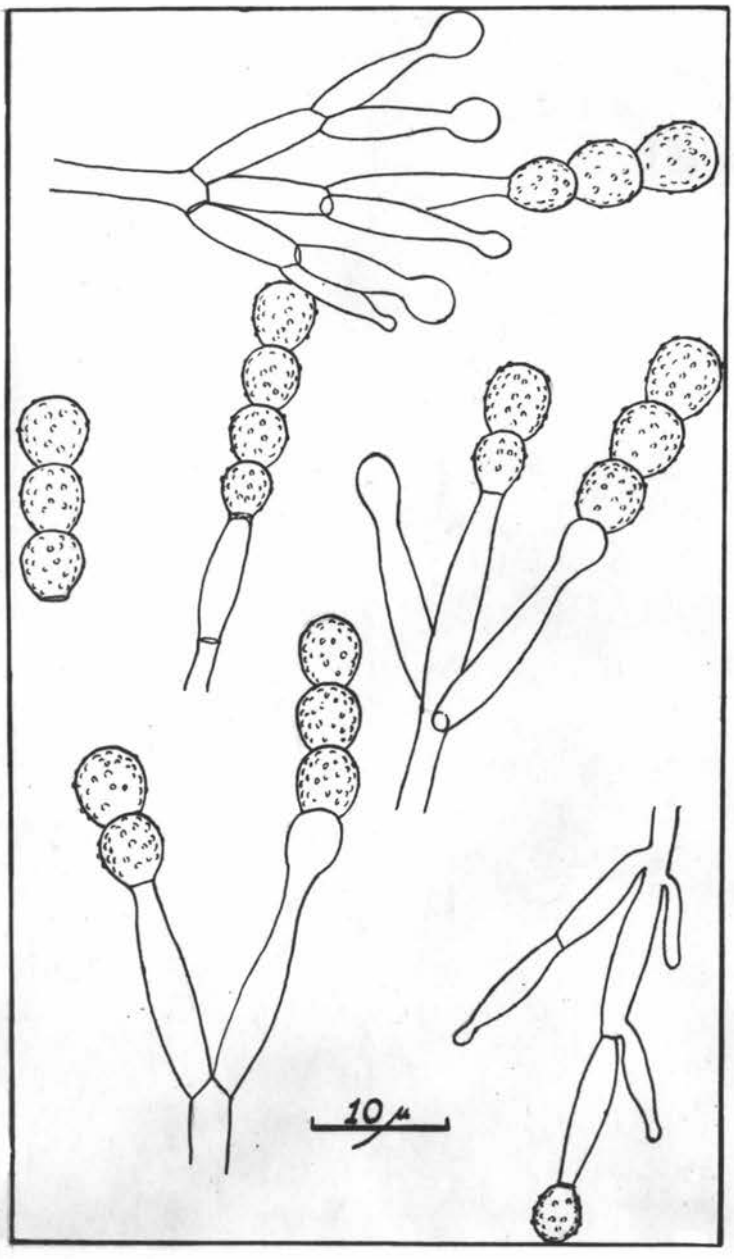

Figure 32. Scopulariopsis brevicaulis (Sacc.) Bainier. Hyaline, verrucose conidia and conidiophores.

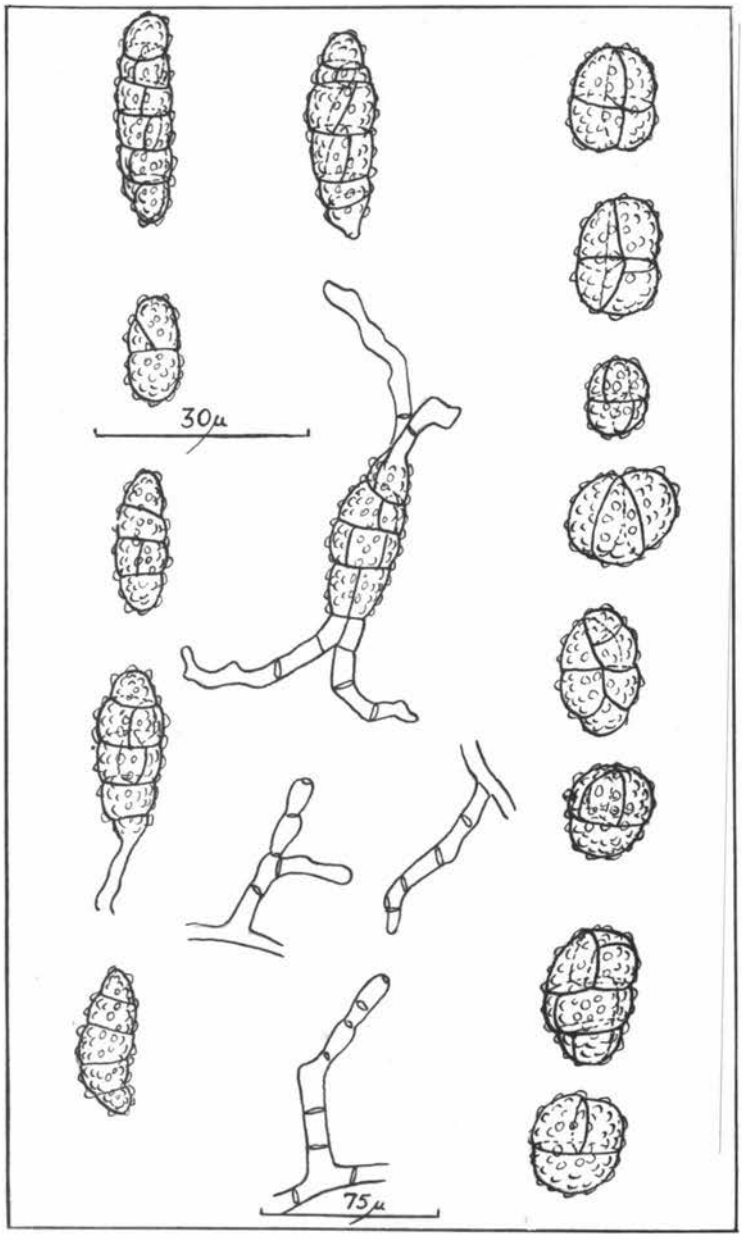

Figure 33. Stemphylium consortiale (Thum.) Groves. Conidia, conidiophores and a single germinating conidium. Conidia brown to almost black in color.

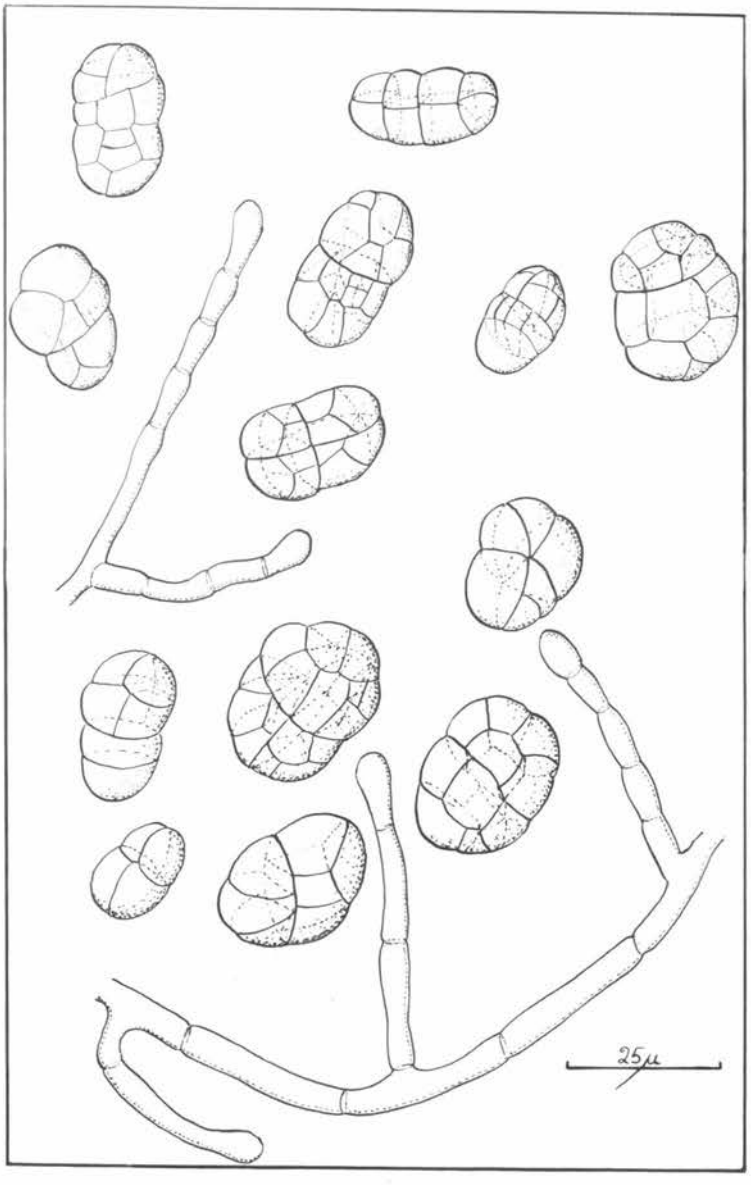

Figure 34. Stemphylium sarcinaeforme (Cav.) Wiltshire. Smooth, olivaceous brown conidia and conidiophores.

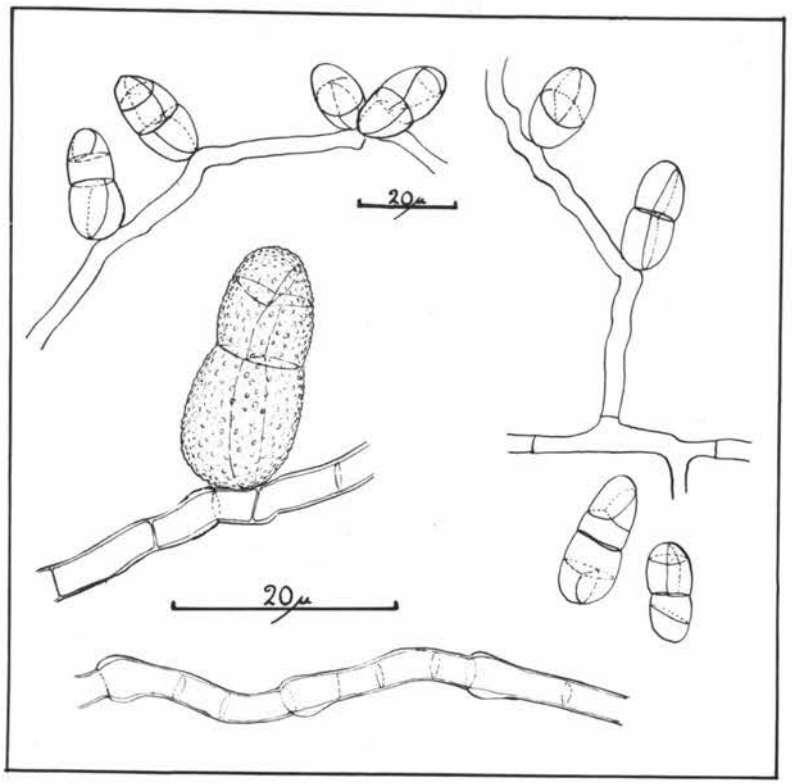

Figure 35. Stemphylium sp. (Pseudoplea?). Left center) Verrucose, olivaceous brown conidium. Lower) Conidiophore on which conidia have been borne terminally and then pushed to one side as the conidiophore has extended. Remainder) Typical growth of conidia and conidiophores.

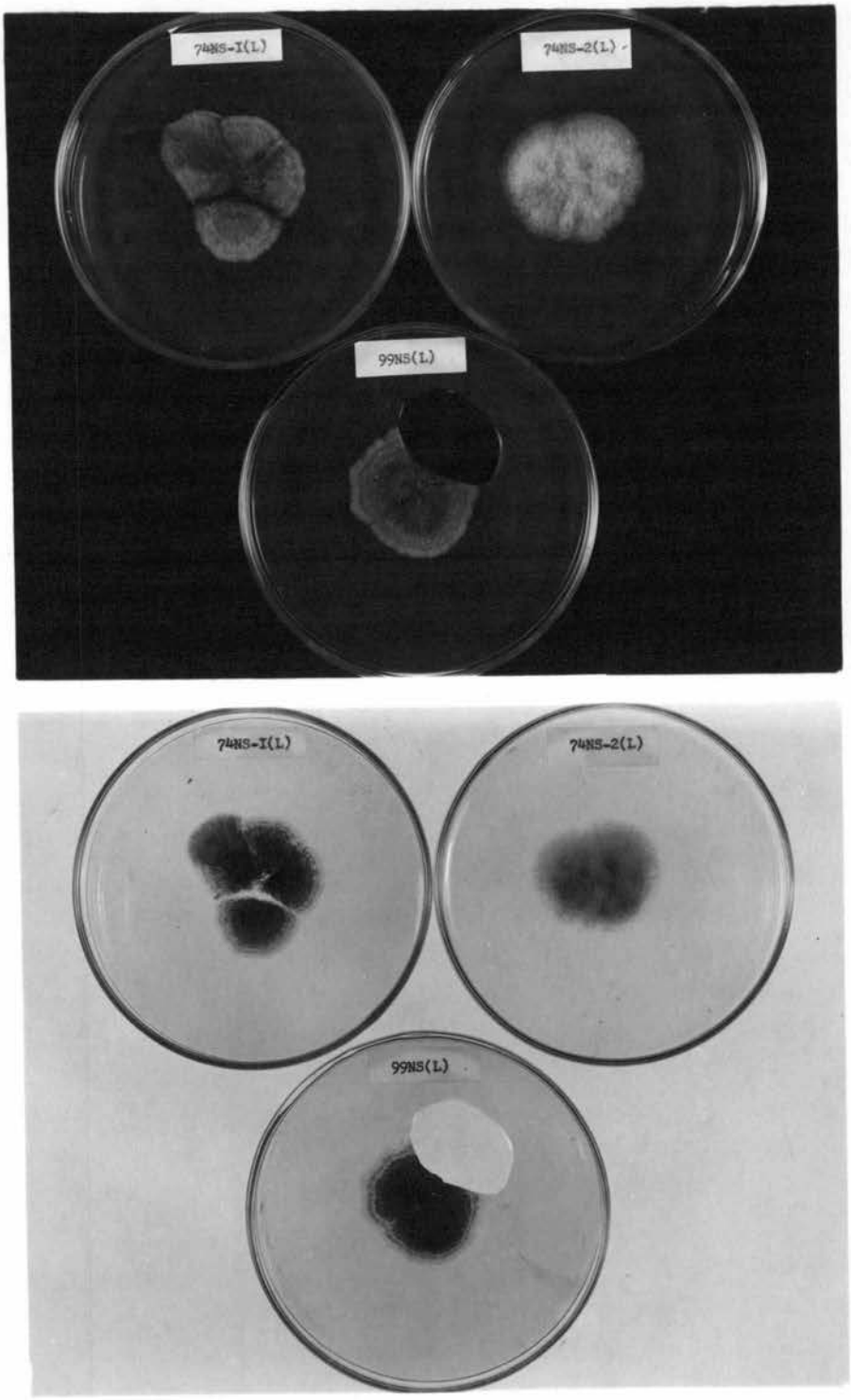

Figure 36. Stemphylium sp. (Pseudoplea?). Ten day old cultures grown on malt agar at 21°C. Upper) Cultures photographed under transmitted light, showing the production of minute sclerotia or incipient fruiting bodies. Lower) Photographed under reflected light.

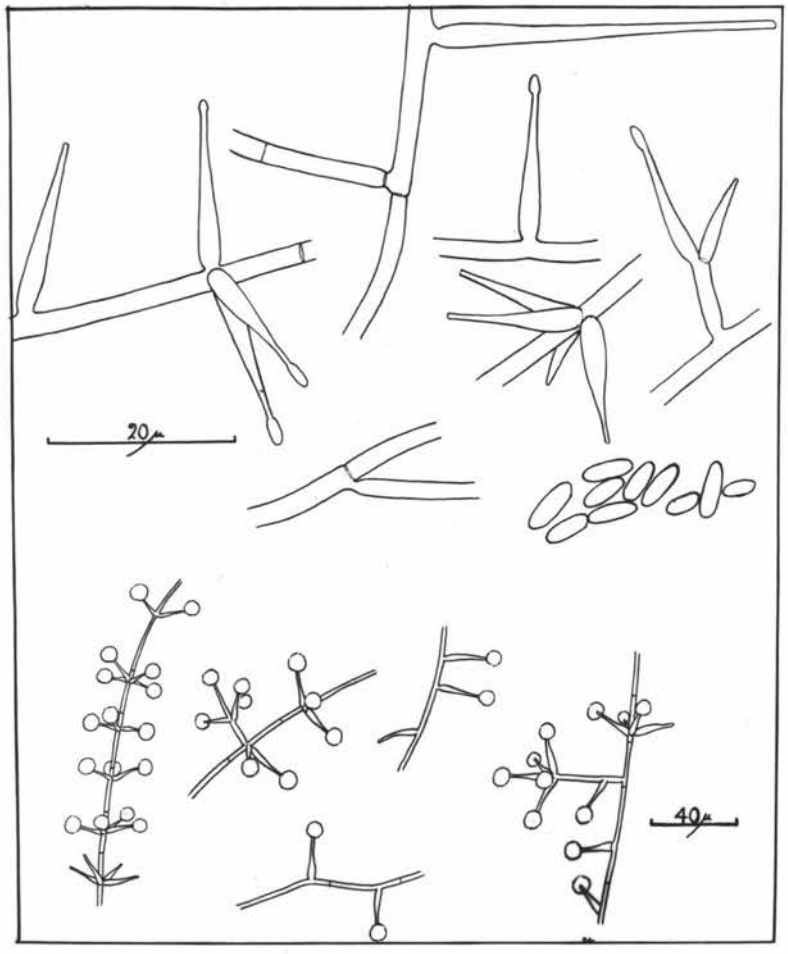

Figure 37. Verticillium sp. Lower) Hyaline conidiophores and globules of conidia. Upper) Conidiophores, conidia and hyphae.

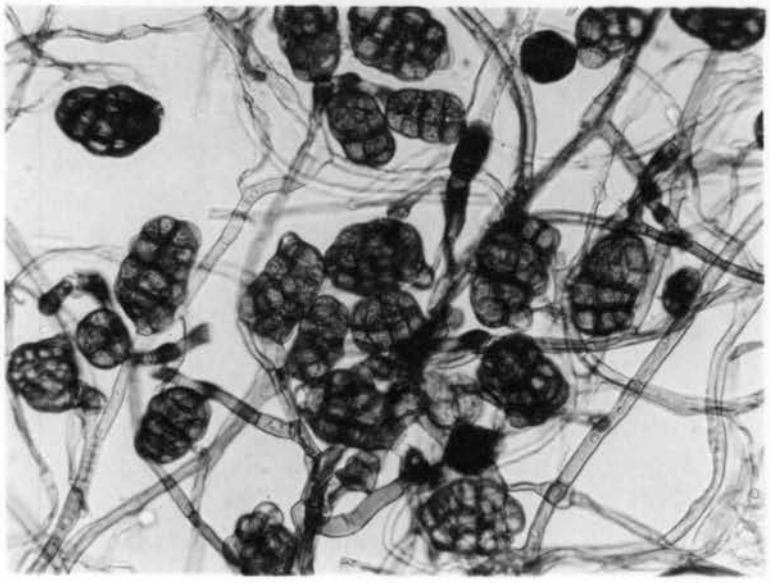

Figure 38. Stemphylium botryosum Wallroth. Conidia and hyphae. x400.

Figure 39. Didymium difforme (Pers.) Gray. Sporangia surrounding a disintegrated red clover seed on a germination blotter. x19. (Photo. by F. P. McWhorter)

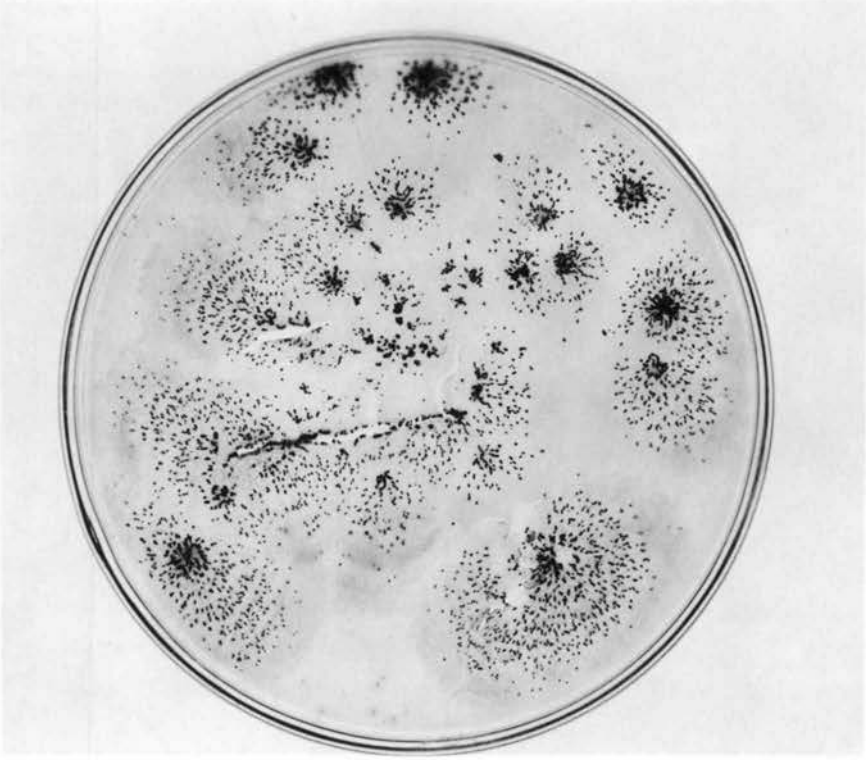

Figure 40. Penicillium novae-zeelandiae van Beyma. Ten day old culture on malt agar showing abundant formation of black sclerotia. x1.

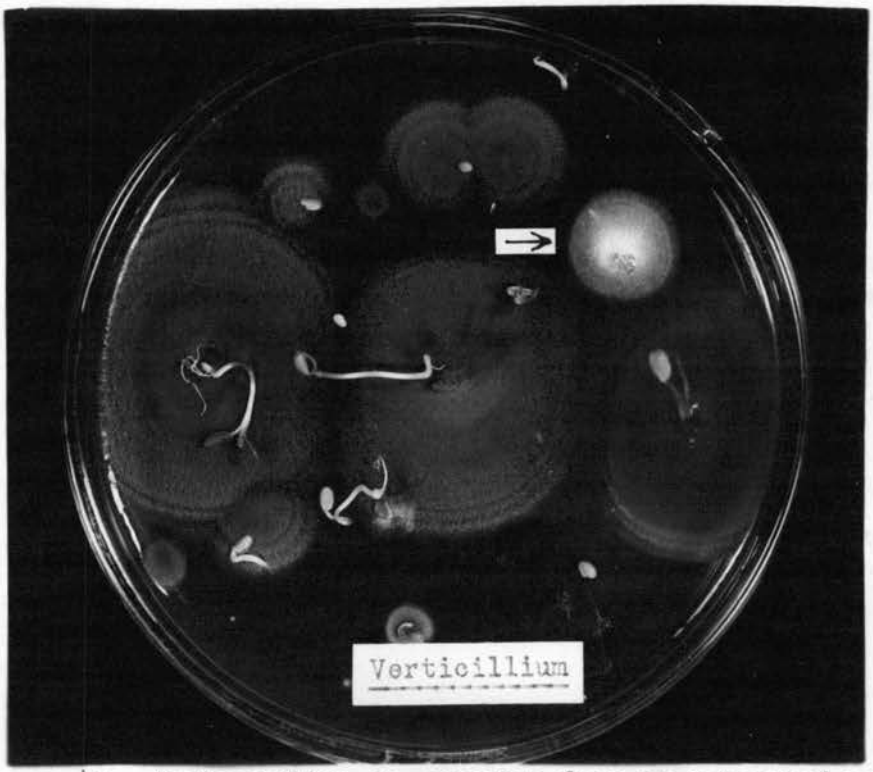

Figure 41. Verticillium sp. growing from an untreated red clover seed plated on malt agar.