EVALUATION OF RESISTANCE TO SCLEROTINIA CROWN AND STEM ROT CAUSED BY SCLEROTINIA TRIFOLIORUM IN SELECTED ALFALFA CULTIVARS

by

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Thesis submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE
in
Plant Pathology, Physiology, and Weed Science

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January, 1988

Blacksburg, Virginia

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(ABSTRACT)

Sclerotinia crown and stem rot (SCSR) incited by *Sclerotinia trifoliorum* Eriks. causes severe losses in some fall-seeded, no-tillage plantings of alfalfa (*Medicago sativa* L.) in Virginia. A mycelial plug inoculation technique was used to detect differences between cultivar (cv) responses of two alfalfa cvs, Arc and Vertus, under greenhouse conditions. A six dia plug from the margin of a 5-day-old culture of *S. trifoliorum* was placed near the crown area of a plant and incubated for a pre-determined period in a dew chamber at 18 C and 100% RH. Differences in isolate virulence were detected; cv Vertus was less susceptible than Arc to the less virulent isolates while the more virulent isolate (TAL 4) was equally severe on both. An incubation period of 96 hr produced significantly higher disease severity than 72, 48 or 24 hr, however, cv differentiation was best after 72 hr. Eight-, and nine-week-old plants were found to be most suitable for cv evaluation tests since

younger seedlings were severely damaged and more mature plants did not develop sufficient symptom expression. Evaluation of twelve cvs with the virulent isolate (TAL 4) and the less virulent isolate (LAL 3) after 96 hr incubation produced significant differences between the mean disease severity ratings (MDSRs). Disease severity increased up to 20 days and then stabilized. Cultivar Anstar followed by WL 320, Vertus and Saranac AR were less susceptible in a majority of the tests; Endure and Euver performed well in some tests while Pioneer Brand 526 and Raidor performed poorly in all tests. This inoculation technique may act as the primary step in the selection of disease resistant germplasm for propagation, re-evaluation, and mass selection before field testing.

Dedicated

To The Memory

Of

My Late Parents

LYMAN S. AND ELIZABETH D. KULATHUNGAM

ACKNOWLEDGEMENTS

The author wishes to express gratitude and appreciation to the following people.

1. Dr. Erik L. Stromberg, for his willingness to serve as Committee Chairman. The author greatly appreciated his guidance, instruction, encouragement, enthusiasm, patience and untiring support. His confidence and trust in the author were encouraging. The author also wishes to express gratitude for having been given the opportunity to work on on the *Sclerotinia* project. The author wishes him well in his career.

2. Dr. Stephen W. VanScoyoc, for his willingness to serve as a Committee Member. His enthusiasm, guidance, and help in the planning and conduct of the research project were very appreciated. The author found his comments on the contents of this thesis very useful. The author wishes him well.

3. Dr. Martha K. Roane, for her willingness to serve as a Committee Member. Her gentle advice, very helpful criticism, patient instruction and excellent editing in the preparation of the material in this thesis were appreciated. The author wishes to express gratitude for her friendship and help at all times.

4. Dr. Larry D. Moore, and the Department of Plant Pathology, Physiology, and Weed Science of VPI & SU, Blacksburg, VA, for having given the author the opportunity and the financial assistantship to do her Master's Degree.

5. Dr. Gail Tomimatsu, for her unselfish and willing assistance in the analysis of the data. The author appreciated very much her encouragement, patience, instruction and most of all sharing of her expertise and time.

6. Dr. Erik Smith, and Mr. Brian Marx, of the Statistics Department, for their willingness to help in the analysis of the data. The author appreciated this very much.

7. Mr. Stanley Long, for his assistance with various operations in the greenhouse. His consent to help at all times was appreciated and it enabled the author to successfully complete all the experiments.

8. Mrs. Karen L. Specht (Nee Reed), for her encouragement, friendship, help and useful discussions on the thesis topic. The author appreciated the opportunity of having worked along with her on this project and wishes her well.

9. Dr. Larry Specht, for his help in learning the Mainframe computer.

10. Mr. Mike Williams, for his willingness to help in the greenhouse. The author wishes to express her gratitude and wish him well.

11. Mr. Brian Smith, for sharing his valuable time to help the author with some of the data analysis. The author wishes him well in his career.

12. Ms. Mary Ann Hansen, for having given the opportunity of gaining work experience in the Plant Disease Clinic, in the Summer of 1987.

13. Messers Ramon Cu, and Bill Chism, for helping the author learn the computer graphics packages.

14. All my friends and collegues in the Department of PPWS, for their kindness, help, understanding and friendship.

15. Mrs. Mary Rhoades for her friendship and assistance in many ways.

16. My late parents, Lyman and Elizabeth Kualthungam, for having given the author the best of education, strenghth of character, immense love, a very happy home and their excellent example of simple, God fearing lives. My mother, for her loving letters and prayers until her demise.

17. My four brothers, Devu, Dharman, Jeyam, Rajan, and their families for thier love and encouragement.

18. My husband, Jeyakumar, for his unselfish act in enabling the author to be apart for so long, and for his very high confidence and belief in her capabilities. The author owes it to him for what she is now. The author wishes to thank him for his love, trust, patience and encouragement.

19. My son Jey (Suthan), for his love, patience, understanding, encouragement, companionship, little sacrifices and the joy of his childhood during this period.

20. My Lord and Saviour for His Everlasting Love, Great Blessings, and Abundant Grace.

TABLE OF CONTENTS

CHAPTER

1 LITERATURE REVIEW

Introduction and Research Objectives 1

Occurrence 4

Disease Cycle and Epidemiology 5

Disease Management Strategies 9

Screening for Resistance 14
Ascospore inoculation 16
Inoculation with sclerotia 18
Inoculation with infested grain 19
Inoculation with mycelium 21

Literature Cited 28

2 DEVELOPMENT OF A MYCELIAL PLUG INOCULATION TECHNIQUE FOR EVALUATION OF ALFALFA CULTIVARS FOR RESISTANCE / SUSCEPTIBILITY TO SCLEROTINIA CROWN AND STEM ROT CAUSED BY SCLEROTINIA TRIFOLIORUM

Introduction 35

Materials and Methods 38

Results 45

Discussion 65

Literature Cited 72

3 EVALUATION OF RESISTANCE TO SCLEROTINIA CROWN AND STEM ROT CAUSED BY SCLEROTINIA TRIFOLIORUM IN SELECTED ALFALFA CULTIVARS IN THE GREENHOUSE

Introduction 74

Materials And Methods. 76

Results 83

Discussion 110

Literature Cited 120

4 SUMMARY 122

APPENDIX A HYGROTHERMOGRAPH TEMPERATURE AND RELATIVE HUMIDITY RECORDINGS DURING THE 1987 GREENHOUSE EXPERIMENTAL PERIOD. 129

APPENDIX B EFFECTS OF DIFFERENT TYPES OF WATERING ON THE DISEASE SEVERITY ON AN ALFALFA CULTIVAR INOCULATED WITH A VIRULENT ISOLATE OF SCLEROTINIA TRIFOLIORUM. 133

VITA 139

Chapter 1

Literature Review

Introduction and Research Objectives

Crown and stem rot of forage legumes caused by *Sclerotinia trifoliorum* Eriksson was reported in Germany in 1857 (20) and in the United States in 1890 (7). Alfalfa (*Medicago sativa* L.) was not included as a host until 1915 (24). Sclerotinia crown and stem rot (SCSR) caused by *S. trifoliorum* is widespread and since that time has been considered a common and destructive disease on forage legumes, especially clovers (*Trifolium* spp.) and alfalfa, in temperate regions of the world such as Great Britain, Czechoslovakia, Denmark, Estonia, Germany, Holland, Poland, Italy, Sweden, Russia and in the western hemisphere in parts of United States of America and Canada (2, 15, 40, 49, 50). Significant losses in alfalfa due to SCSR occur in cool, humid areas including Great Britain, Norway, Sweden, Canada and the United States particularly in the northeastern and far western states (15, 67, 74). Sclerotinia crown and stem rot is reported to be severe on alfalfa in the eastern United States during winter and early spring when there is snow cover or cool wet weather (26).

Alfalfa is an important forage in Virginia and in recent years numerous cases of significant stand loss or complete stand failure in fall-seeded alfalfa has occurred in the spring following no-tillage planting. Sclerotinia crown and stem rot caused by *S. trifoliorum* has been determined to be the cause (65).

This disease is widespread in Virginia and is perpetuated by the presence of clovers and other small seeded legumes. When sods containing such hosts are used for no-tillage planting of alfalfa in the fall, the crop is at risk from SCSR (66). Alfalfa is frequently attacked in its first year, sometimes so severely that a complete stand failure occurs but it appears to have increasing resistance or tolerance after one season's growth. Fall plantings that have withstood the first year are seldom severely affected in succeeding years (15). Alfalfa plants of all ages are susceptible to *S. trifoliorum* but the incidence and severity are greatest in seedlings. The damage may vary from season to season and is often scattered within plantings (15, 67, 75, 76). In the spring, damage in an alfalfa field appears as areas of dead plants varying in size from a few square centimeters to the entire field (59, 75, 76). Sclerotinia crown and stem rot disease on alfalfa can be easily recognized by the scalded patches of dead and dying seedlings in affected fields (2, 21). Van Scoyoc and Stromberg in 1984 (70) estimated that on the basis of an average establishment cost of about $500 per hectare an economic loss of $303,400 had occurred on the previous fall's alfalfa crop in Virginia due to SCSR. Recently severe stand decline problems in alfalfa have been identified in Otsego county in New York, in association with Verticillium wilt and SCSR (5).

At present there are no economically feasible management strategies for the control of SCSR in no-tillage plantings of alfalfa. The use of disease resistant cultivars will be

benificial to any integrated disease management program. No alfalfa cultivar of agronomic importance is known to be totally resistant to SCSR. It is thus important to have a reliable and repeatable cultivar screening technique by which large numbers of plants could be evaluated under greenhouse conditions, prior to field tests. This may act as a 'sieve' in detecting resistant germplasm which then would be useful in obtaining resistant breeding lines.

Research Objectives

The research objectives of this study were :

1) to develop an efficient, repeatable, artificial inoculation technique that will allow differentiation between resistance/susceptibility levels of alfalfa cultivars and germplasm to attack by *S. trifoliorum* under controlled greenhouse conditions.
2) to evaluate alfalfa cultivars and germplasm for resistance / susceptibility to SCSR using the inoculation technique developed in objective 1.

Occurrence

Sclerotinia trifoliorum grows best in cool wet weather. It is a widely distributed pathogen and occurs in most countries where clover or other small seeded legume crops are grown. It has been reported from nearly every country of Europe as well as from Russia and the North American continent (15, 40). The prevalence and intensity of SCSR on clover, is known to depend on weather conditions. Clover rot on clover appears to be worse after a mild winter (13, 15) and disease spread is favored by periods of mild, damp weather (16). *Sclerotinia trifoliorum* survives as sclerotia in the soil or on the surface of the soil in infested residues during summer months (15, 67). Loveless (40) has shown that active mycelium growth of the pathogen takes place only within a humidity range of approximately 95-100% RH, growth being completely inhibited at about 90.2% RH. Environmental humidity subsequent to infection is important in *S. trifoliorum*, for here the mycelium is not entirely endophytic but spreads on the surface of the host. Some researchers have stressed the importance of very high relative humidity for rapid development of the disease (37, 45, 60). Since aggressive infection and mycelium growth after ascospore germination occur over a wide temperature range, 0-20 C (37), -2-27 C (39), or 5-20 C (40), but only within narrow limits of humidity, rainfall may be expected to be more critical than temperature in the epidemiology of clover rot(40). *Sclerotinia trifoliorum* possesses the ability to grow and attack susceptible clover

plants at an optimum of 15-18 C (37, 47). The fungus as mycelia is killed at temperatures of -24 C and 42 C (39). *Sclerotinia* species can tolerate a wide range in pH but are best adapted to an acidic substrate the optimum being 5.5 (47, 77). *Sclerotinia* species can also grow readily in or on a medium of basal salts of essential elements and a simple carbon source. Growth of *S. trifoliorum* was shown to be better on ammonium than on nitrate nitrogen (77).

Disease Cycle and Epidemiology

Much that is known about the disease cycle of *S. trifoliorum* is from reports of SCSR on clover, also referred to as clover rot. However, the salient features of this cycle are similar for alfalfa (Stromberg, unpublished). The pathogen survives the summer months as sclerotia which are black and round to irregular in shape (74) and range from 0.3 to 10 mm (53, 80). About 90% of the life cycle of *Sclerotinia* spp. is spent in the soil as sclerotia (1) that are viable in soil for 3 to 8 years (15, 38, 47, 79). Most sclerotia are formed on or in the stem bases and around the crowns of colonized plants. There is no precise information on the survival of sclerotia of *S. trifoliorum* in soil but soil temperature, moisture, organic content and microbial activity are likely to be determining factors (40, 58). No evidence is available that sclerotia of *S. trifoliorum* produce mycelia in the soil (33). Even if the sclerotia did produce any mycelia, the hyphal growth observed by William and Western (78) was very sparse and extended only a limited distance from the

parent sclerotium. Thus it is unlikely that any significant amounts of infection in the field takes place in this manner (14, 15 33, 40). Some workers consider that in clover mycelia infection is more important than that from ascospores (22, 23, 24, 73, 80). During summer the sclerotia remain dormant but in the fall, those near the surface give rise to apothecia (13, 15, 58, 79). A single sclerotium may produce one or several apothecia, pinkish to buff in color, saucer shaped, 2 to 8 mm dia, and borne on stipes of varying lengths. In recent studies in Virginia by Reed (57) greatest numbers of apothecia were produced during the months of November and December in microplots of alfalfa infested artificially with sclerotia. She found that greatest number of apothecia occurred between 5 C and 10 C and during the season that received most rainfall. Ascospores from apothecia are forcibly discharged, carried by air currents and upon successful infection and colonization of susceptible host plants under suitable conditions, cause leaf spots in clovers (15, 58, 69, 74). A symptom of initial infection of affected clover plants, the inconspicuous peppering of leaves and stems with small dark brown or black spots, may escape notice. Primary infection occurs during cool, wet weather. Infection in clovers is favored by high humidity, mild temperatures and freedom from excessive water on leaf surfaces (15, 37). In late winter or early spring, foliage and stems become yellow, turn flaccid and finally collapse (67). The crown and basal portion of the infected stems may become soft and discolored (21, 26).

Secondary infection occurs as mycelia from ascospore incited lesions on infected leaves grow out on the leaf surface and spread by contact to neighboring leaves, and invade tissues of the petioles and stems (74). In alfalfa and particularly in no-tillage seedling alfalfa, the mycelia can spread easily along the ground or drill-strip and infect adjacent plants at the crown causing wilt and death of plants (15, 40, 58, 70). The affected plant parts or the soil may be covered with mycelia (67). Although *S. trifoliorum* may kill the foliage rapidly in clover, it does not destroy the roots quickly. New buds may thus form and lead to a partial recovery (15, 61). This may be the case in alfalfa as well. However, in heavily infested fields, large patches of plants may completely disappear and weeds may colonize the bare ground (15). The secondary infection and spread of SCSR may continue throughout the winter and spring until warm (21 C or above) dry weather reduces growth of the fungus (15). At this time, the mycelium forms sclerotia in or on the dead stems, crowns and on the roots near the surface (17, 21, 67). Sclerotia remain dormant in soil through the summer and provide a source of primary inoculum the following autumn, thus completing the disease cycle (17, 26). (FIG.1).

Sclerotinia trifoliorum infects crimson clover by direct penetration as a result of a break in an epidermal cell caused by mechanical pressure from the infection cushion, destruction of the cuticle and the epidermis by enzymatic action, growth of hyphae through stomata (very rarely) or a combination of these

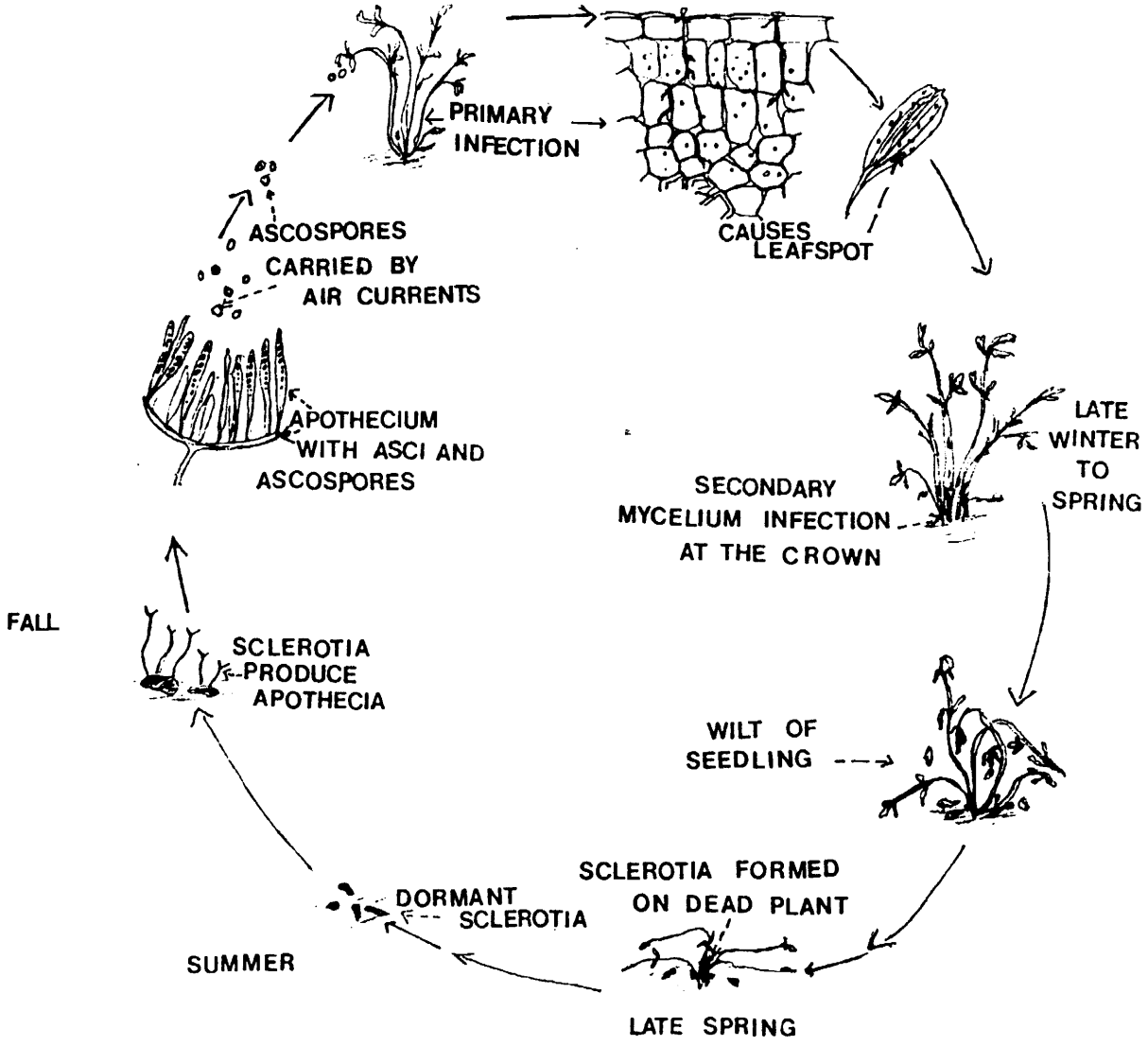

Figure 1. Disease Cycle Of Sclerotinia trifoliorum On Alfalfa

methods (51). Prior and Owen (51) also reported the presence of several layers of decayed cells below an infection cushion of *S. trifoliorum* on Buffalo alfalfa. Purdy(52) suggested that ascospores may produce a diffusible substance that entered the clover leaves through stomata and disorganized the cells prior to penetration by mycelium of *S. trifoliorum*. Maceration of clover tissues by pectolytic enzymes and oxalic acid produced by *Sclerotinia* spp. has been reported (3, 77). Increased levels of oxalate and polygalacturonase activity have been observed in clover tissue diseased by *Sclerotinia* spp.(43). Not only does oxalic acid affect pH of the infected tissue but it is toxic to host cells and may also be responsible for wilting symptoms in clover associated with disease caused by *Sclerotinia* spp.(29).

Disease Management Strategies

Most diseases caused by *Sclerotinia* spp. have not been controlled consistently and economically (64). The facts that *S. trifoliorum* produces successful survival structures, the sclerotia, and large numbers of air-borne ascospores which can be disseminated to long distances, has allowed *S. trifoliorum* to be widespread and a successful pathogen wherever forage legumes are grown. Seed infested with mycelia, or contaminated with sclerotia have the greatest potential for dissemination of *Sclerotinia* spp. over long distances (1, 15). Mycelia of *S. trifoliorum* have been reported on white clover seed though not identified possitively due to dessication of seeds (62).

Factors such as the type of crop, method of cultivation and

environmental conditions influence the effectiveness of management practices for control of plant disease. Several strategies for management of SCSR in alfalfa have been suggested. In general, any factor which minimizes the amount of foliage during the period of ascospore discharge of the pathogen or which prevents conditions of high relative humidity, will tend to check the development of the SCSR (15).

Control practices that have been employed for the management of SCSR in alfalfa fields include both cultural practices and chemical methods. Crop rotation is an essential management strategy for the control of any soil borne disease. Crop rotation of alfalfa and other small seeded legumes with non-susceptible crops have given only partial control (58). The facts that sclerotia can survive for many years even in the absence of a host (47), and that ascospore infection can occur from nearby heavily infected forage legume crops have reduced the effectiveness of rotations. Crop rotation to control SCSR in clover has been observed to be only moderately successful (40, 64). *Sclerotinia trifoliorum* has been associated with many weed spp. belonging to the families such as Cruciferae, Caryophyllaceae, Geraniaceae, Leguminosae, Compositae, Boraginaceae, and Plantaginaceae. This may explain the longivity of *S. trifoliorum* in the field (15). Investigation of possible weed hosts to this pathogen in fall-seeded, no-tillage alfalfa fields in Virginia should prove interesting and most useful in better understanding of the disease. A study in this area is

proposed (Stromberg, personal communication).

Grazing by sheep or clipping off of top growth of alfalfa in late fall to allow the soil surface to dry rapidly and thus retard the spread of the pathogen have been shown to reduce disease build up (58). However, autumn grazing must be practiced with discretion in clover since the resulting severe diminution in plant establishment due to grazing or cutting could outweigh the loss caused by *S. trifoliorum* especially in poor thin land. Grazing / clipping may be effective only in years of very luxuriant crop growth (40) or when conducted after a killing frost.

Continuous flooding of a field for 23-45 days or a cycle of alternate flooding and drying of field prior to planting, though found effective in the control of *S. sclerotiorum* (44), is impractical (77). Welty(74) proposed deep plowing to bury the sclerotia. Cook et al. (9) reported that deep plowing to a depth of 25 cm did not affect disease severity of white mold of bean (*Phaseolus vulgaris* L.) caused by *S. sclerotiorum* in Nebraska. Such practices are not suitable in no-tillage alfalfa fields.

Wide-row spacing of plants helped reduce canopy density and white mold disease on bean (11). Reed (57) proposed that the presence of *S. trifoliorum* may pose a threat to any plant population density in alfalfa fields. However, she suggested that a wider row spacing should reduce spread of SCSR in alfalfa. Effects of field orientation on disease incidence and

severity have been indicated by Bennet & Elliot (4) who reported differences in incidence and severity of forage crown rot caused by *S. trifoliorum* on north- and south-facing slopes. Similar observations have been made by Hass and Bowlyn, on bean white mold severity in Canada (27).

Adjusting the date of planting of crops to avoid severe disease loss has been one of the management strategies in the control of many diseases. Since ascospores, which are the primary source of inoculum in SCSR in the field, are discharged from the apothecia only during fall, (at most times), this management practice should prove beneficial. Dillon-Weston (15) reported that alfalfa crop planted in the spring was not affected by SCSR caused by *S. trifoliorum*. Reed (57), in a plant age study in alfalfa in Virginia, determined that mid- to late-summer planting of alfalfa should be avoided if the field is known or suspected to be infested.

Many chemicals have been tried to reduce the ascospore infection of SCSR in clover and alfalfa. Copper and sulphur compounds were used but with inconclusive results (73). Jenkyn (31), stated that benomyl foliar sprays applied monthly from September to January increased yield of red clover and decreased the number of apothecia of *S. trifoliorum*. Welty and Rawlings (76) reported the efficacy of one timely foliar application of benomyl in the control of SCSR on seedling alfalfa. Pentachloronitrobenzene (PCNB) has been found to be effective on the soil surface in infested alfalfa fields (58).

Other chemicals such as cyanamide (64, 77) dazomet (77), 2, 6-dichloro 4-nitroaniline (DCNA, 42), dichloran (77), and vinclozonin (32) have been used on a variety of crops for the control of *Sclerotinia* rots. Chemical control, however may not be found to be economically or practically feasible for some field crops (40). Repeated applications of specific chemicals within a growing season or succession of seasons may give rise to tolerant strains of *Sclerotinia* spp. though none has yet been reported for *S. trifoliorum* (64).

The existence of potential for biological control of *Sclerotinia* spp. has been investigated. Fourteen species of fungi belonging to the genera *Acrostalagmus, Trichoderma, Fusarium, Gliocladium, Hormodendrum, Mucor, Penicillium,* and *Verticillium* were described as parasitic on sclerotia of *S. trifoliorum* based on *in vitro* tests (41). Turner and Tribe (68) reported a 65% reduction of sclerotia of *S. trifoliorum* in the field by soil application of a dust preparation of *Coniothyrium minitans* pycnidia. However, no disease control studies have been attempted. Since microbial activity on sclerotia is one of the determining factors of the survival of sclerotia of *S. trifoliorum* in the field, biological control may become increasingly important.

The necessity of developing resistant cultivars of forage legumes, especially alfalfa for fall, no-tillage establishment, for the successful and economical management of SCSR is clear. The use of resistant cultivars along with cultural practices

involving modification of microclimate most suitable to the location appear to be the most useful disease control measures for field crops (64).

Screening for resistance

The goal of most alfalfa breeding programs is to incorporate resistance to as many economically important diseases and insect pests as possible and yet maintain the desirable agronomic traits needed for high yielding cultivars. The use of resistant cultivars not only increases yield and forage quality but also reduces the need for expensive chemical control (34). The production of resistant cultivars appears to be desirable since at present there are no satisfactory alternative control methods (40). More work has been done on the evaluation of resistance to SCSR with clover cultivars (13, 33, 36) than with alfalfa. In recent years, resistance levels in alfalfa cultivars have been investigated by some workers in Europe (55, 61) and in the United States (18, 75). Researchers have described differences in reaction of some clover and alfalfa cultivars to SCSR. Some cultivars and plants within cultivars of these two species sustain less SCSR damage than others (2, 10, 13, 16, 18, 28, 46, 50, 51, 54, 69, 75). However, resistant cultivars of agronomic importance are not currently known (2, 19, 48, 74). Under long and heavy infection periods all cultivars succumb to SCSR (13). Although differences in disease resistance can be detected between cultivars, the plant breeder needs to detect the more subtle

differences found within a plant population (48). Evaluation of resistance to SCSR of forage legumes in the field has been observed to be difficult because the disease often occurs erratically among and within plots and its incidence and severity vary from year to year (13, 50). Also in experiments carried out under natural field conditions the severity of SCSR is largely dependent on the weather (13, 15). In some years the disease may be absent altogether (40). Under natural field conditions it might be impractical to test large numbers of plants under uniform infection pressure (54). In such tests for evaluation of resistance, it is important that reliable and easily repeatable techniques be developed, whereby large numbers of plants can be evaluated uniformly and quickly. Cultivar screening experiments for SCSR resistance under natural field infestation should be conducted with a low overall disease incidence (15, 75). Researchers have stressed the importance of screening experiments with a sufficiently low disease level to enable distinguishment of differences between cultivars. Selection for resistance to SCSR can be hampered by either too little disease resulting in many escapes or by too much disease resulting in an abnormally heavy attack and death of entire population (13, 75). Carr (6) observed the necessity to devise a method that would act as a preliminary 'sieve' in the selection of constant types for cultivar building i.e. breeding lines. Alfalfa is cross-pollinated and relatively heterozygous and this makes it difficult in obtaining homozygous resistant

material.

Several artificial inoculation techniques have been developed and used for evaluating resistance of clover and alfalfa cultivars and plant introductions to SCSR both in the field and in the greenhouse. The difficulty of producing an artificial epidemic of *S. trifoliorum* for the purpose of such studies has been pointed out. It is, however, desirable and necessary to produce symptoms and disease severity on the test plants as similar as possible to those in nature (75) because this will prove to be essential in differentiating between disease responses of cultivars tested.

The choices of a suitable inoculum and inoculum dosage are most important for such studies. Different types of inocula of *S. trifoliorum* have been used in pathogenicity as well as cultivar evaluation experiments, both in clover and to a lesser extent in alfalfa.

Ascospore inoculation

Ascospores are the source of primary infection in the field (40, 69, 77) and have been used by a number of workers to infect clover plants (8, 36, 39, 56). Keay (33) found that, while ascospores liberated from apothecia produced by sclerotia buried around the base of plants caused infection of clover plants, clover leaves sprayed with an aqueous suspension of ascospores were not infected. Wadham (73) failed to obtain infection with ascospores in pure water unless the clover leaves were wounded or a small amount of nutrient was added to the ascospore

suspension. Nicolaisen et al. (45) brushed ascospore suspensions on leaves of clover plants which produced leaf spots, but the disease failed to develop further. Loveless (40) was able to induce ascospore infection by suspending sclerotia bearing mature apothecia about 6 mm above the test clover plants kept over water under bell jars. Although he observed differential leaf spotting between plants, he could not control the spore load. Dijkstra (13) observed that although ascospore infection without rotting produced a dense cover of brown leaf spots on 6 cultivars of red clover known to be of different susceptibility to SCSR, there was no significant difference in the density of spots between cultivars, an indication that this stage of infection was not suitable for varietal evaluation. In experiments comparing the ascospore inoculation technique with a mycelium technique, Dijkstra could not demonstrate that the former was better for selection of resistance to SCSR in red clover cultivars. She also found that ascospore inoculation should always be preceded by an artificial weakening of plants such as frost damage, whereas mycelium inoculation produced the symptoms of rotting of plants without prior wounding. In later work it was shown that freezing of clover plants at -6 C or -8 C before ascospore inoculation favored spread of rotting caused by mycelium from ascospore lesions and the feasibility of this technique for SCSR screening work was suggested (14). In recent years Raynal (54) used different concentrations of ascospores with or without additions of glucose (10g per liter + Tween®20)

to facilitate attachment to the epidermis) to inoculate detached leaves from two month old clover plants and observed necrotic lesions but no rotting of leaves. He also determined that a concentration of 10^4 ascospores per ml best differentiated between cultivars. Although cultivar response to ascospore infection exists and closely approaches natural infection in the field, the production of large number of ascospores of *S. trifoliorum in vitro* for such purposes is not yet practical (36, 54, 69).

Inoculation with sclerotia

The fact that sclerotia are the survival structures of *S. trifoliorum* which give rise to ascospore-producing apothecia and the ease with which they are produced in large numbers both in culture and in the field, led several workers to investigate the feasibilty of using sclerotia as a source of inoculum for pathogenicity as well as for cultivar screening experiments with clover. Wadham (73) failed in his attempt to infect clover plants grown in pots by burying sclerotia at varying depths. Keay (33) failed to obtain infection with sclerotial fragments buried in sand around test plants. Rudorf (60) used atomized aqueous suspensions of "sclerotium flour" from sclerotia produced in culture to inoculate foliage of clover plants and obtained infection in 3 to 6 weeks. Nicolaisen et al. (45) used a modified technique with dried, pulverized sclerotia to inoculate clover plants and obtained infection but with unreliable results. Kreitlow (36) used sclerotia from different

isolates of *S. trifoliorum* to infect clover plants but mycelium failed to grow from the sclerotial fragments. Schmidt (61) found that scattering of pulverized sclerotia at the base of clover plants gave irregular results and thus under these conditions the method is unreliable. This method was observed to be unsuitable for differentiating cultivars(54). Scott and Evans (63) reported that of 36 sclerotia placed adjacent to seedlings of red clover 12 of them did develop a mycelium which infected the seedlings.

Inoculation with infested grain

Grain infested with actively growing mycelium of *S. trifoliorum* has been used as a source of inoculum for the purpose of screening clover cultivars for resistance to SCSR both in the field and in the greenhouse studies. Good field infection was obtained by scattering of infested grain, prepared by growing the pathogen on a medium made up of 2:1 v/v wheat and oats, over plots of clover plants (47). Cormack (10) obtained good infection by placing cultures of the pathogen grown on oat-hull medium in contact with roots and crowns of clover plants in the field. Carr (6) dusted clover seedlings with cultures grown on bran, dried and powdered. Though this inoculum could be preserved at 3 C to 4 C for considerable periods of time without loss of viability, the survival rate of the seedlings was poor. Kreitlow (35, 36) used a modified technique of Pape which employed moist or dry infested grain to inoculate the crowns or stolons of greenhouse grown clover

plants and obtained excellent infection within 24-72 hr under favorable temperature and moisture conditions. However, a number of shortcomings with infested grain inoculum are (i) difficulty in timing preparation of inoculum with proper growth stage of test plants, (ii) lumping of fresh moist grain inoculum, (iii) production of an abnormally heavy attack of disease and (iv) rapid killing of plants (13, 36, 75). Graham and Hanson (25) used the dried-grain inoculation technique of Kreitlow (36) for inoculation of red clover plants in the greenhouse and found that factors such as age of host, moisture, and temperature influenced the results obtained. These could be limitations in the use of this technique in the greenhouse. However, they obtained good infection on red clover plants in the field by scattering dried grain inoculum with a suitable inoculum dosage during fall. The feasibility of this technique in screening of large numbers of clover plants for resistance to SCSR was indicated.

Elgin and Beyer (18), using an inoculum consisting of a composite of 12 *S.trifoliorum* isolates grown on dried grain, tested 6-week-old plants of 82 selected alfalfa clones and polycross progeny of 71 of these under controlled growth chamber conditions. Their results indicated that although all clones and their progeny were susceptible to attack significant differences in their susceptibility existed among them. Results obtained in the study were cosidered unreliable since the most resistant lines were susceptible, the results were statistically

undesirable, and significant differences were observed among replications inoculated with different batches of dried grain inoculum. Nevertheless the authors stated that this method might be useful in selecting for resistance in the field (18).

In North Carolina field plot experiments, varietal differences to *S. trifoliorum* could be observed among 17 and 23 alfalfa cultivars in two consecutive years in a naturally infested field. However, the seedlings of both field resistant cultivar Appalachee and susceptible Victoria succumbed to the disease when inoculated with infested dried grain inoculum and placed in a mist chamber. It was concluded from this study that improved greenhouse selection methods are essential for SCSR screening (75).

Inoculation with mycelium

Although the mycelium produced by the sclerotia of *S. trifoliorum* has not been determined to be the source of primary inoculum in the onset of SCSR in either clover or alfalfa under field conditions, some early workers believed that mycelium infection was important. Gilbert and Meyer (24) stated that during winter and early spring the fungus exists as a saprophyte and then as a parasite upon the host plants, and when ascospores germinate they send out among the soil particles mycelial threads which infect young alfalfa or clover plants on contact. After the first warm days in February, growing hyphae may be found on the surface of the soil in the field and, soon

after, infection of the host plants may be observed (23). Wolf and Cromwell(80) also believed that the vegetative mycelium at or near the ground level is reponsible for the disease and that little disease results naturally by direct infection with ascospores in clovers. Wadham (73) reported that a saprophytic mycelium stage of *S. trifoliorum* must first be established before living tissue can be attacked. Hino (30) suggested that the primary source of infection may be mycelium arising from the sclerotia in the soil or mixed with seeds. These workers stressed the importance of the mycelium phase of the disease cycle in the actual development of SCSR. The development of the mycelium inoculation technique has been helped by the fact that *S. trifoliorum* can be isolated and cultured in its vegetative form on a number of media (54).

Keay (33), in her pathogenicity test with clover, used pieces of inoculum cut from the edge of actively growing cultures of *S. trifoliorum* on malt extract or oatmeal agar, placed them on non-wounded surfaces of the test plants and observed the development of diseased tissue. In breeding for resistance to clover rot Frandsen (22), Nuesh (46), and Vestad (71) used cultures of *S. trifoliorum* grown on extracts of clover. Stolons of Ladino clover under natural field conditions were frequently attacked by *S. trifoliorum* at the nodes, internodes and terminal bud. Mycelium was ocasionally observed spreading over the surface of the soil from diseased stolons. In early spring, infection apparently spread from infected stolons to

adjacent healthy ones either through the soil or through detritus on the surface. Based on these observations, Kreitlow (35) suggested that most effective artificial inoculation of Ladino clover at least, would probably result if the inoculum was placed directly in contact with the stolons or crowns of test plants. His inoculation technique of scattering pieces of agar grown culture of the pathogen on the soil among Ladino clover plants in flats of steamed soil failed to produce satisfactory infection and the agar was quickly overgrown by contaminants (36). Vestad (71) mixed mycelia of 15 isolates from different locations in Norway and sprayed a suspension of this in a clover decoction with 2% fructose and obtained differential disease reactions between the tetraploid and diploid cultivars of clover. Nicolaisen et al. (45) used a solution of *S.trifoliorum* grown on malt extract to inoculate foliage of clover plants.

In recent years, some screening work on clover and alfalfa has been done in Sweden and France (54, 61). Schmidt placed cotton pads moistened with a mycelium suspension of *S. trifoliorum* in contact with the base of each stem of clover plants and incubated them under conditions of low temperature, reduced light and high humidity without condensation. A highly significant classification of cultivars was obtained. Two main factors which appeared to influence plant resistance were: the ability to slow down the pathogen growth rate and the ability of the host to regenerate (61). Raynal(54) observed that foliar

spraying of inoculum made from mycelium fragments of *S. trifoliorum* in water or a nutrient medium produced unreliable results on clover cultivars. Although a rapid and simple technique, this could not be used to differentiate between cultivars for the following reasons (i) presence of hyphal fragments of different ages in the homogenate of mycelia which varied from test to test, (ii) poor sticking of the inoculum droplets to the foliage in spite of the use of a surfactant in the inoculum suspension, (iii) unequal dispersion of inoculum on the collars and (iv) the presence of untimely rotting due to saprophytes which grew in the nutrient medium (54).

Raynal (54) thus developed a mycelial plug inoculation technique, very similar to the one used by Keay (33) in her pathogenicity tests. When plugs from the margins of 3-day-old vigorously growing cultures of *S. trifoliorum* isolates on solid media, without the presence of sclerotia, were used to inoculate clover plants, variability within and between experiments was reduced. Raynal (54) deposited such plugs on to the non-wounded collar region of clover cultivars. To test the validity of this inoculation technique, he used two month old greenhouse grown alfalfa plants. His preliminary experiments showed that disease was more severe on two month old clover plants than on 7 month old plants. The inoculated plants were kept in a dew chamber at 18 C and at a relative humidity approaching 100% for a period of 96 hr. The plants were then removed to the greenhouse (22-28 C) and watered only enough to avoid wilting. Disease symptom

ratings were taken on a scale of 1-3 at about 10 days after inoculation (1 = Plant without symptoms, 2 = plant showing wilt and collar necrosis, 3 = dead plant). Using this technique, Raynal observed that the results obtained were reliable because in all of his tests, the field resistant clover cultivar Tetri was the least attacked; similar results were seen with two alfalfa cultivars, Ancre and Magali, which showed high resistance. The advantages of this mycelial plug inoculation technique are all test plants can be (i) inoculated in an identical manner, (ii) at the same inoculation site, (iii) with a constant quality and quantity of inoculum and (iv) rapid results can be obtained. However, an effective control of moisture in and around the test plants is required after removal from the dew chamber (54).

Raynal(55) also evaluated 12 *Medicago* selections and 13 cultivars of red clover with the above technique and determined that *Medicago sativa* cvs Vertus and Euver were highly resistant and *M. rugosa* Desr. and *M. scutellata* Mill. were found to be immune and *M. tornata* Mill. and *M. truncatula* Gaertn. were resistant.

In techniques involving the foliar application of inoculum such as ascospores, sclerotium flour or mycelium suspensions, a period of usually 3-8 weeks is required to obtain reliable information concerning the relative resistance or susceptibility of plants being tested. This is because the pathogen must grow downward through the petiole and stem in order to attack the

crown. Until recently, no selection has been undertaken in alfalfa for resistance to SCSR. Since the problem has been determined to be intense in fall-seeded, no-tillage alfalfa, rapid methods for evaluation of resistance in alfalfa would be very useful to the breeder. Raynal's mycelial plug inoculation technique (54) was shown to be valuable for distinguishing responses of red clover and may do the same for alfalfa. However, a proper rating system to detect subtle differences between cultivars should be investigated.

Other inoculation methods which have been tried include bud-cell suspensions from shake cultures of *S. trifoliorum* used as a root-dip or a foliar spray on detached seedling leaves and petioles of clover plants (17) grown in the greenhouse. Results obtained were neither reliable nor reproducible. They also looked at the feasibility of counting the number of sclerotia around infected plants, measuring lengths of healthy and diseased rows and the use of an arbitrary diagrammatic assessment key, to describe the levels of disease severity among 19 cultivars of clover, under natural field conditions. The use of an assessment key was found to be the most useful and could be applied to any type of trial layout (17).

Evaluation of resistance to oxalate and mycelia of *S. trifoliorum* was carried out on alfalfa cultivar Arc. Plants resistant to different levels of oxalic acid were selected and later tested under controlled temperature and moisture conditions in the greenhouse for resistance to *S. trifoliorum*

grown on wheat kernels. The selection for resistance to oxalate was not a satisfactory procedure for improving resistance to *S. trifoliorum* (59).

The physiology of *Sclerotinia* disease resistance has not been studied adequately. However, three general types of resistance reactions to *Sclerotinia* spp. have been described (i) resistance of tissue to breakdown possibly associated with nutrition of the fungus, (ii) presence of preformed antifungal materials and (iii) formation of phytoalexins. Resistance of clover to *S. trifoliorum* has been attributed to the more efficient use of food reserves by certain clover varieties resulting in a resistant middle lamella that is less easily hydrolyzed by enzymic action (69). Antifungal activity toward *S. trifoliorum* has been shown for 7-hydroxy-4' methoxy-isoflavone from clover (72). Two clover cultivars resistant to *S. trifoliorum* were found to accumulate more phytoalexin than the susceptible ones, though the final overall concentration on the resistant ones would not have inhibited the growth of *S. trifoliorum in vitro* (12).

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Chapter 2

The Development of a Mycelial Plug Inoculation Technique for Evaluation of Alfalfa Cultivars for Resistance / Susceptibility to Sclerotinia Crown and Stem Rot Caused by *Sclerotinia trifoliorum* Eriks.

INTRODUCTION

In recent years alfalfa (*Medicago sativa* L.), an important forage in Virginia, has been reported to suffer significant stand losses due to Sclerotinia crown and stem rot (SCSR) caused by *Sclerotinia trifoliorum* Eriks. in fall-seeded, no-tillage plantings (13, 14, 15). The occurrence of many biotypes within *S. trifoliorum* has been demonstrated (3, 9, 10). Kreitlow (7) reported that different monoascospore cultures of *S. trifoliorum* varied distinctly in pathogenicity to Ladino clover (*Trifolioum pratense* L.). Marked variations in growth among normal isolates of *S. trifoliorum* have been reported (10). Kreitlow (7) also reported 4 cultural types of *S. trifoliorum* from single ascospores which differed in growth rate and ranged from non-sclerotial types to those that produced abundant sclerotia on potato dextrose agar (PDA). Different isolates of *S. trifoliorum* may also differ in their pathogenicity to alfalfa cultivars (cvs). *Sclerotinia trifoliorum* attacks clover plants over a wide range of temperature of 0 C to 27 C, the optimum being 15 C to 18 C and at a relative humidity of about 90% or more (9).

Alfalfa is a heterogeneous crop which is pollinated by bees

and it is difficult to obtain homozygous resistant material. Several artificial inoculation techniques have been developed for evaluating resistance of clover, and to a lesser extent, of alfalfa to SCSR, both in the field and in the greenhouse. Ascospores, the primary inocula in the onset of SCSR under natural field conditions, may be the most desirable source of inoculum for use in artificial screening techniques; however, there are many difficulties to be overcome before ascospores can be successfully used to screen germplasm. Differences to ascospore infection exist between clover cvs, but the production of large number of ascospores of *S. trifoliorum in vitro* has been found to be difficult (8, 11). Inoculation of clover plants with either sclerotia or infested grain gave neither reliable nor reproducible results (2, 12, 17). Several workers failed to obtain infection of clover plants with sclerotia or sclerotial fragments buried around plants or with foliar inoculation with atomized aqueous suspensions of 'sclerotium flour'(5, 8, 11, 12). A number of shortcomings in the use of infested grain inoculum, such as (i) lumping of fresh moist grain inoculum, (ii) production of abnormally heavy attack of disease, and (iii) rapid killing of plants were pointed out (1, 17). When Elgin and Beyer (2) used infested grain inoculum to inoculate alfalfa cvs to select for resistance, they obtained unreliable results since both field resistant and susceptible cvs succumbed to the disease. Though the mycelium phase of infection is reported to be secondary to ascospore infection in the course of SCSR in

clover and alfalfa under field conditions, some workers have used culture suspensions or plugs of *S. trifoliorum* grown on solid medium to inoculate these two species, in pathogenicity and cultivar evaluation tests (5, 7, 11, 12, 16). Schmidt (12) placed cotton pads moistened with a suspension of *S. trifoliorum* in contact with the base of each stem of individual clover plants, incubated them under low temperature and high humidity conditions and obtained significant differentiation of cultivars. Raynal(11) developed a mycelial plug inoculation technique in which he placed plugs from the margins of 3-day-old cultures of a *S. trifoliorum* isolate on the non-wounded collar region of clover cultivars and incubated them in a dew chamber at 18 C and humidity approaching 100% for 96 hr. He obtained reliable and rapid results in the evaluation of some red clover cvs, a few alfalfa cvs, and other *Medicago* species.

The objective of this study was to investigate the suitability of adopting a mycelial plug inoculation technique to differentiate between the resistant and susceptible responses of two alfalfa cultivars to *S. trifoliorum*. This objective was carried out in different parts, (i) investigation of differences in virulence of isolates of *S. trifoliorum* on two cultivars of alfalfa, (ii) determination of a suitable incubation period of the inoculated plants in the dew chamber for adequate infection and symptom development, (iii) determination of a suitable age of seedling for inoculation in such screening studies. The findings of this objective will be used in carrying out

objective 2 in which selected alfalfa cvs will be evaluated for resistance to *S. trifoliorum* using the mycelial plug inoculation technique.

MATERIALS AND METHODS

All of the inoculation experiments were conducted in a greenhouse at VPI & SU, between March and July of 1986.

The isolates of *S. trifoliorum* used in this study were from a collection of large single ascospore isolates obtained from apothecia produced by field collected sclerotia, maintained by Ms. Fran Svrcek in 1984-1985. *Sclerotinia trifoliorum* is dimorphic in ascospore size with four large and four small ascospores within the same ascus (6). The isolates were confirmed as those of *S. trifoliorum* by Dr. Linda Kohn (University of Toronto) in March 1985. The isolate groups were coded as SAL (from Sinks farm, Montgomery County, Va), CAL (from Crockett farm, Wythe County,Va), TAL (from VPI & SU Dairy farm, Montgomery County,Va), and LAL (McCoy farm, Rockbridge County, Va). Stock cultures of eleven isolates belonging to the four groups were maintained on PDA slants at 20 C in dark in an incubation chamber and working cultures were transferred at frequent intervals to PDA and incubated under the same conditions. Differences in growth rate of the eleven isolates were observed by transferring 6mm plugs from the margins of young cultures to the center of the PDA in Petri (9cm) dishes and measuring the diameter (dia) of the colony at 24 hr intervals. There were three replicates per isolate. The

isolates whose colony dia did not reach the periphery of the Petri dish at the end of 5 days were noted. Five isolates, namely LAL 3, CAL 1, CAL 3, TAL 2, and TAL 4, were selected for the preliminary inoculation experiment because they were (i) slow to moderate in growth on PDA at five days i.e., the colonies did not cover the medium in the Petri dish and (ii) sclerotia were absent at five days. Differences in the colony dia of the 5 isolates selected were also determined in a separate study. The number of sclerotia produced per PDA plate by the eleven isolates were determined. The colony growth of the eleven isolates were also tested at an incubation temperature of 28 C. The isolates used for inoculation studies were all grown at 20 C on the same batch of PDA (Difco) per experiment.

In a preliminary experiment, the disease responses of two alfalfa cvs, Arc (commonly grown American cv) and Vertus (an European cv reported as resistant to SCSR by Raynal, 11), were tested with five isolates, namely, LAL 3, CAL 1, CAL 3, TAL 2 and TAL 4 respectively. Plants of cvs Arc and Vertus were grown two-months under greenhouse conditions in plastic Cone-tainers®(Ray Leach Cone-tainer® nursery, Canby, OR 97031) containing a mixture of steam pasteurized soil, and peat with vermiculite (1:1:1), lime (to adjust pH to 6.0) and Rhizobium inoculant (for nodulation). Three seeds per Cone-tainer® were seeded but later thinned at twelve days to one seedling. Two days after thinning, plants were fertilized with P and K in

in accord with Virginia soil test recommendations (1 ml / Cone-tainer® of a solution of 13.1g KCl and 19.6g Na_2HPO_4 per liter). Plant media were brought to saturation prior to inoculation.

Six mm plugs were cut with a sterile corkborer from the outer edges of 5-day-old actively growing cultures of the five isolates and a single plug of an isolate was placed on the non-wounded area of the crown of a single plant of both cultivars. The plug was pressed down so that it touched all stems of the plant. Two sets of 39 replicates per cultivar per isolate with 2 non-inoculated control plants per treatment were randomly arranged in trays that were then placed in a dew chamber maintained at 18 C and relative humidity approaching 100%, both of which are favorable conditions for mycelium infection and disease establishment. One set of treatments was incubated in the dew chamber, for 48 hr and the second set for 96 hr. At the end of the respective incubation periods, plants were transferred to the greenhouse bench. Two days after removal from the dew chamber plants were bottom-watered by placing the trays of plants for 1 minute daily in a larger plastic tray containing a standard volume of water (26 liters). At no time after inoculation were the plants top watered during the period of the experiment. This type of watering was used to avoid enhancing the activity of the pathogen while at the same time keeping the plants sufficiently moist to prevent water stress.

Disease severity ratings of individual plants in all treatments were taken on a simple scale of 1-3 as proposed by Raynal (11), 1 = plant without any disease symptoms, 2 = plant with wilted stems and or stem necrosis, 3 = dead plant. The observations were recorded at 5, 8 and 12 days after inoculation. Date of inoculation was March 14, 1986. Based on the criterion that there was no significant difference between the pathogenecities of CAL 3 or CAL 1 or between TAL 2 and TAL 4 on the two alfalfa cvs tested in the preliminary experiment, three isolates (LAL 3, CAL 3, and TAL 4) were selected for further inoculation studies.

The experiment (with 3 isolates) was repeated to confirm the earlier observations, to test the reliabilty of the technique, and to select a suitable incubation period of the plants after inoculation, that would permit sufficient symptom development without killing all plants. All plants were grown in the same soil mix (soil:peat:vermiculite, 1:1:1), lime, *Rhizobium* inoculant and with metalaxyl (Apron® 25 W, Ciba-Geigy) used as a soil drench to prevent damping-off caused by *Pythium* spp. Four sets of twenty five, two-month-old greenhouse grown plants of each cv Arc and Vertus were inoculated individually with plugs of mycelia from 5-day-old cultures of isolates LAL 3, CAL 3, and TAL 4. The four sets of inoculated plants (25 / cultivar /incubation period /isolate) and the non-inoculated control plants (1 / treatment) were completely randomized within trays which were randomly placed in the dew chamber and incubated for four

different periods, 24 hr, 48 hr, 72 hr and 96 hr, respectively, at 18 C and 100 % RH. The treatments were then removed at appropriate intervals from the dew chamber and arranged on the greenhouse bench, which received supplemental light from a high pressure sodium vapor lamp (1000 watt, HPSV approximately 4 ft. above the plants). The treatments were bottom watered daily starting 2 days after removal from the dew chamber. Disease severity ratings were recorded at 5, 10 and 15 days after inoculation. In this experiment a rating scale of 1-7 was adopted because it was found to be more appropriate than the 1-3 scale used before since a range in the degree of wilt per plant within cvs was observed. The total number of stems per plant was also recorded to assist in determining the percentage wilt per plant. The rating scale used was 1 = plant without symptoms, 2 = plant with wilted stem with no necrosis, 3 = plant with stem necrosis and 0-25 % stems wilted, 4 = plant with stem necrosis and 26-50 % stems wilted, 5 = plant with stem necrosis and 51-75 % stems wilted, 6 = plant with stem necrosis and 76-100 % stems wilted but not dead, 7 = plant dead. Experiment 1 was inoculated on June 16, 1986, and Experiment 2 on June 20, 1986.

The third part of this study involved determining the most suitable age of plants which can be used in the mycelial plug inoculation technique when screening for disease resistance / suceptibility. The purpose of this was to prevent severe disease from killing all the plants or insufficient disease to

distinguish disease responses between cultivars.

Two experiments (Age study 1A and 1B) were carried out in which two sets of six age groups, namely 4- 9- week-old greenhouse grown plants of the two cvs Arc and Vertus, were inoculated with plugs from 5-day-old cultures of isolate TAL 4 and were incubated for 48 hr and 96 hr respectively. The experimental design and the procedure were essentially similar to the one described earlier. However, these experiments had less number of replicates per treatment due to poor growth of the test plants. The plants exhibited phytotoxicological symptoms manifested as marginal scorch, chlorosis of leaves and stunting as a result of metalaxyl toxicity. Age study 1A had 20 inoculated plants per treatment while 1B had uneven sample size, with 17 being the maximum. Age study 1A was inoculated on July 16, 1986, and 1B on July 21, 1986. Both sets were completely randomized. After removal from the dew chamber, the respective treatments of both experiments were kept on the same greenhouse bench which received light from a 1000 watt HPSV lamp for 9 hours. Plants were bottom-watered daily and disease severity ratings were recorded at 10, 15, and 20 days after inoculation based on a rating scale of 1-6: 1 = plant symptomless, 2 = plant with stem necrosis and 0-25% stems wilted, 3 = plant with stem necrosis and 26-50% stems wilted, 4 = plant with stem necrosis and 51-75% stems wited, 5 = plant with stem necrosis and 76-100% stems wilted but not dead, 6 = dead plant. A score was not given for the symptom of wilted stem without necrosis since it

was not observed in any of the inoculated plants.

In another study (Age study 2) on the effect of plant age on SCSR disease response the influence of three isolates, (LAL 3, CAL 3 and TAL 4) were tested on older plants of alfalfa cvs Arc and Vertus. Two sets of eighty-five-day-old greenhouse grown plants were cut back, fertilized and regrown for 21 days. The plant media were brought saturation and then inoculated with plugs of the three isolates individually as described earlier. One set was incubated for 48 hr and the other for 96 hr in the dew chamber under the same temperature and humidity conditions as before and later removed to the greenhouse bench which received HPSV light for 9 hr. The treatments were bottom--watered as before and the disease severity ratings were recorded at 5, 10 and 15 days after inoculation. Each treatment had 50 plants /cultivar /isolate / incubation time with 1 non-inoculated plant per treatment as control. In this experiment the 1-3 rating scale was adopted, since the range of symptoms produced was very narrow. The date of inoculation of age study 2 was May 29, 1986. In all these experiments, the respective isolates were reisolated on to PDA from necrotic areas and from wilted stems of living plants and from dead plants. Data of all experiments except that of age study 1B (due to lack of sufficient replicates) were statistically analyzed with appropriate analysis of variance procedures (ANOVA) and Student--Newman-Keul's test (SNK) for comparison of treament means (18).

RESULTS

In the laboratory studies comparing the colony size of the eleven original isolates belonging to 4 groups, it was observed that they had a faintly grey or white mycelium on the surface of the media. Significant differences were observed in the colony dia after 96 hr incubation (TABLE 1). After 120 hr incubation (5 days) the colony growth of isolates SAL 2 and SAL 3 had reached the periphery of the Petri dish but this was not observed in the other isolates. Sclerotial initials were observed to develop in about 8-10 days after colony transfer to media. Mycelium tufts are first formed which later begin to aggregate, become pigmented and mature into black sclerotia of irregular shape (size = 2mm-15mm). The rate of maturation of sclerotia among the isolates differed but in an inconsistent manner. However, the isolates LAL 1 and LAL 3 developed sclerotia about 3 days later than the others tested. The pattern of sclerotia formation also did not appear to be very consistent. Complete maturation of the sclerotia occurred within about 10-12 days. Most isolates formed sclerotia in a ring at the edge of the Petri dish, or in a ring a little interior to the periphery or near the center; sometimes sclerotia appeared to be randomly scattered. There was, however, a significant difference in the number of sclerotia produced per isolate after 20 days (TABLE 2). Isolates CAL 1, LAL 3 and LAL 1 produced significantly greater number of sclerotia than the others tested. Microconidia were present on

TABLE 1. Radial growth of eleven isolates of *S. trifoliorum* on potato dextrose agar (BBL) at 20 C after 96 hr incubation.

Isolate	[y]Mean Colony dia (cm)
1. SAL 4	2.47 a[z]
2. LAL 1	3.36 b
3. LAL 3	3.57 bc
4. TAL 4	3.62 bc
5. TAL 2	3.68 bcd
6. TAL 1	3.79 cde
7. CAL 3	3.84 cde
8. CAL 2	3.98 def
9. CAL 1	4.10 ef
10. SAL 2	4.20 f
11. SAL 3	4.20 f

[y] colony dia is an average of three replicates per isolate after 96 hr incubation.

[z] numbers within column followed by the same letter are not significantly different using Student-Newman-Keul's test for comparison of treatment means. ($P \leq .05$).

TABLE 2. Number of sclerotia produced on potato dextrose agar (Difco) per 9 cm Petri dish by eleven isolates of *S. trifoliorum* at 20 C.

Isolate	[y]Mean no. of sclerotia
1. SAL 2	38.7 a[z]
2. TAL 2	39.0 a
3. SAL 3	39.0 a
4. TAL 1	41.0 a
5. CAL 2	45.0 a
6. CAL 3	45.3 a
7. SAL 4	48.7 a
8. TAL 4	53.0 a
9. CAL 1	85.7 b
10. LAL 3	96.3 b
11. LAL 1	102.7 b

[y] average number of sclerotia produced by three replicates per isolate at 20 days after incubation.

[z] numbers within column followed by the same letter are not significantly different according to Student-Newman-Keul's test for comparison of treatment means. ($P \leq .05$).

mycelium grown on Difco PDA. The 11 isolates did not show any visible colony growth on to the medium at 28 C after 72 hr incubation but resumed growth when transferred to a 20 C incubation temperature. The five isolates selected for the preliminary inoculation study had significantly different colony sizes on PDA after 120 hr (5days) incubation at 20 C (TABLE 3). There was hardly any mycelium growth visible after 24 hr incubation. Isolate TAL 4 had significantly greater colony dia than TAL 2, LAL 3, CAL 1 and CAL 3, and those of CAL 1, CAL 3 and TAL 2 than those of LAL 3. During the course of the experimental period, sectoring or the development of abnormal strains were not observed. All the isolates showed growth patterns and sclerotium formation indistinguishable from the original isolates of *S. trifoliorum*.

In the preliminary experiment, the mean disease severity rating (MDSR) produced for Arc or for Vertus were not the same at the two incubation times or with the 5 isolates. After 48 hr incubation isolates LAL 3, CAL 1 and CAL 3 were less virulent than either TAL 4 or TAL 2 on both cvs at the 12-day rating period. In all cases disease severity increased from 5-12 days after inoculation (TABLE 4). Although no significant differences in the MDSRs between the two cvs were obtained in this study, Vertus generally had less damage than Arc when inoculated with isolates CAL 3 or TAL 2 while LAL 3 produced greater damage on Vertus; TAL 4 was severe on both cvs (TABLE 4). The 96 hr incubation time produced higher disease

TABLE 3. Growth of five isolates of *S. trifoliorum* on potato dextrose agar (Difco) at 20 C after 120 hr incubation.

Isolate	[y]Mean Colony dia (cm)
1. LAL 3	3.18 a[z]
2. TAL 2	3.31 a
3. CAL 3	3.85 b
4. CAL 1	4.25 b
5. TAL 4	4.77 c

[y]average colony dia of eleven replicates per isolate.

[z]numbers within column followed by the same letter are not significantly different according to Student-Newman-Keul's test for comparison of treatment means. ($P \leq .05$).

TABLE 4. Mean disease severity rating (MDSR) of cvs Arc and Vertus inoculated with five isolates of *S. trifoliorum* followed by 48 hr incubation in the dew chamber at 18 C and 100% RH.

TREATMENT		DAYS AFTER INOCULATION		
CV	ISOLATE	5	8	12
Arc	LAL 3	[y]1.4 ab[z]	1.5 a	1.6 a
	CAL 1	1.2 a	1.7 ab	1.8 a
	CAL 3	1.3 a	1.6 a	1.8 a
	TAL 2	1.6 b	2.0 b	2.1 b
	TAL 4	1.6 b	2.0 b	2.1 b
Vertus	LAL 3	1.3 a	1.4 a	1.7 a
	CAL 1	1.4 a	1.6 abc	1.8 ab
	CAL 3	1.3 a	1.5 ab	1.7 a
	TAL 2	1.5 a	1.7 bc	2.0 bc
	TAL 4	1.6 a	1.8 c	2.1 c

[y] MDSR of 39 plants per cv per isolate using a 1-3 disease severity rating scale.

[z] numbers within coulmn followed by the same letter are not significantly different according to Student-Newman-Keul's test for comparison of treatment means ($P \leq .05$). Preliminary experiment. Date of inoculation- March 14,1986.

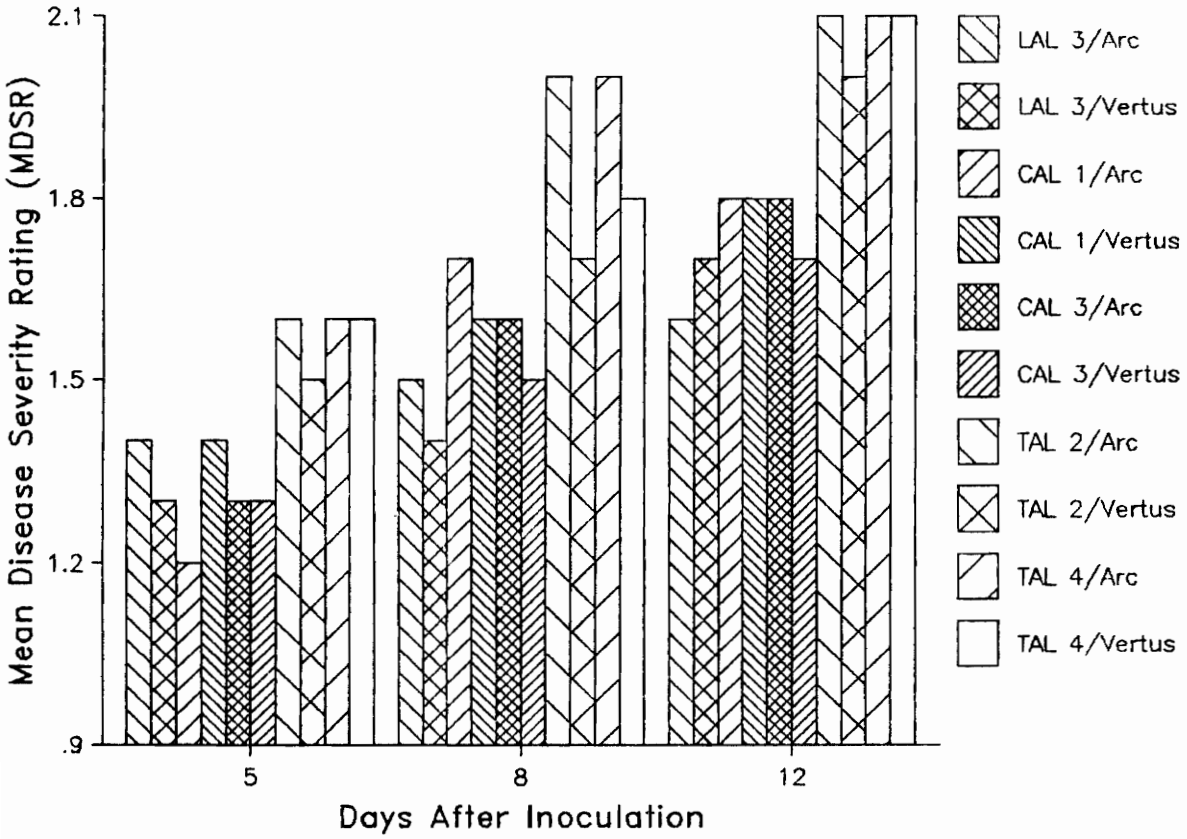

FIG. 2. Disease severity of alfalfa cvs Arc and Vertus inoculated with five isolates of *S. trifoliorum* and incubated in a dew chamber for 48 hr at 18 C and 100% RH.

severity and incidence among all treatments than after 48 hr incubation. However, similar trends were observed after 96 hr incubation; isolates LAL 3 and CAL 3 were less virulent while TAL 4 and TAL 2 caused severe damage on both cvs. Isolates CAL 1, TAL 2 and TAL 4 were more virulent on Arc than on Vertus at the 12-day rating period (TABLE 5, FIG 3). There was a significant cultivar x incubation period x isolate interaction.

The results obtained in experiments 1 and 2 with the three isolates confirmed the above observations. In experiment 1 cv Vertus suffered apparently less damage than Arc at incubation periods of 24, 48, and 72 hr with all three isolates. At 96 hr period, however, isolate caused severe damage on both cvs (TABLE 6). Differentiation between cv responses were observed at all incubation periods, however, it appeared to be best with all three isolates at the 72 hr incubation period. The 96 hr incubation distinguished between the cvs with isolates LAL 3 and CAL 3 but not with TAL 4; TAL 4 was equally severe on both cvs but did not kill all the plants (FIGS. 4-7). Overall disease severity of a cv was dependent on the isolate and the incubation period. Greater the isolate virulence and / or longer the incubation period higher was the MDSR.

The results of experiment 2 were not as desirable as in experiment 1 although the two experiments were conducted under similar conditions, within a week's interval. The same trends were observed with respect to isolate virulence; TAL 4 appeared to be the most virulent. However, the 72 hr incubation produced

TABLE 5. Mean disease severity rating (MDSR) of cvs Arc and Vertus inoculated with five isolates of *S. trifoliorum* followed by 96 hr incubation in the dew chamber at 18 C and 100% RH.

TREATMENT		DAYS AFTER INOCULATION		
CV	ISOLATE	5	8	12
Arc	LAL 3	[Y]1.6 a[Z]	1.6 a	1.9 a
	CAL 1	1.8 b	1.9 b	2.3 b
	CAL 3	1.7 a	1.7 a	2.0 a
	TAL 2	1.9 b	1.9 b	2.6 c
	TAL 4	1.9 b	1.9 b	2.7 c
Vertus	LAL 3	1.7 a	1.7 a	2.1 ab
	CAL 1	1.8 a	1.8 a	2.1 ab
	CAL 3	1.7 a	1.7 a	2.0 a
	TAL 2	2.2 b	2.2 a	2.3 ab
	TAL 4	1.8 a	1.8 a	2.4 b

[Y] MDSR of 39 plants per cv per isolate using a 1-3 disease severity rating scale.

[Z] numbers within column followed by the same letter are not significantly different according to Student-Newman-Keul's test for comparison of treatment means ($P \leq .05$). Preliminary experiment.
Date of inoculation-March 14, 1986.

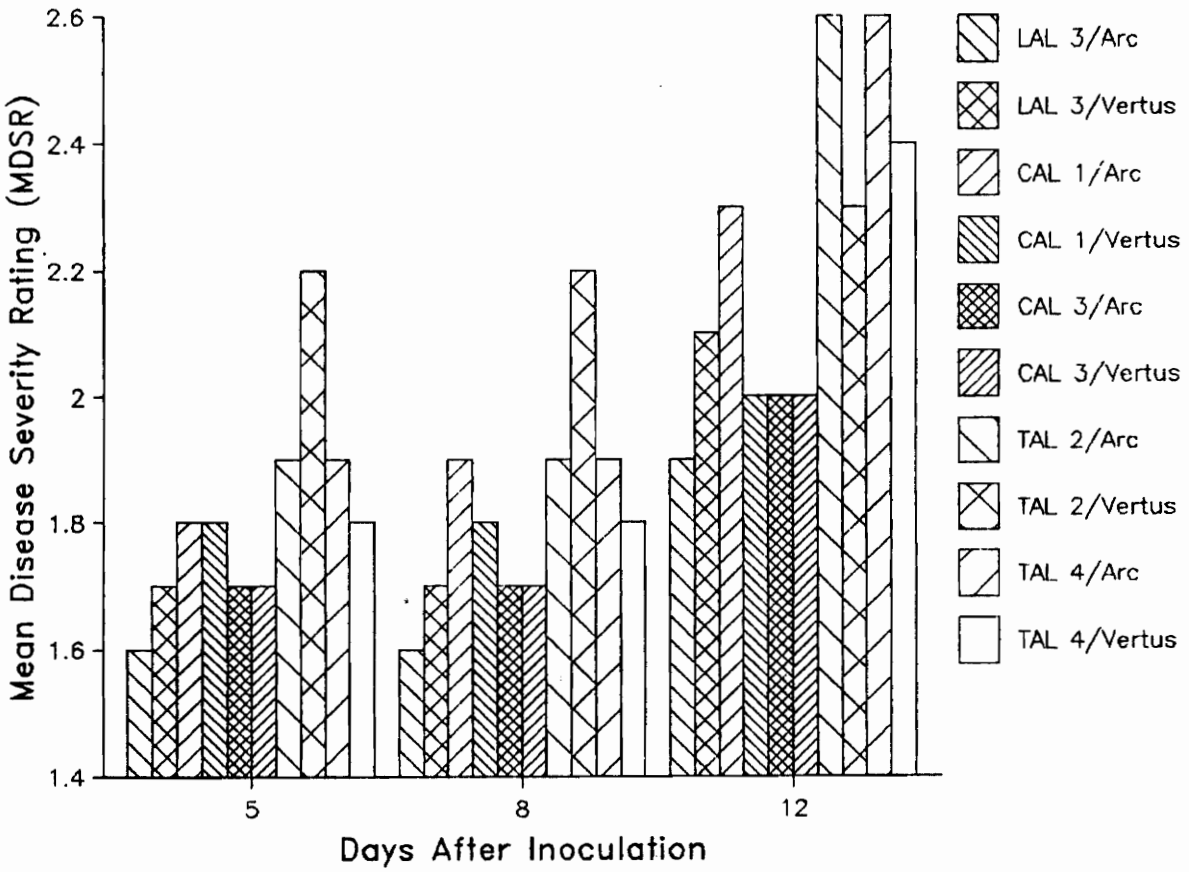

FIG. 3. Disease severity of alfalfa cvs Arc and Vertus inoculated with five isolates of *S. trifoliorum* and incubated in a dew chamber for 96 hr at 18 C and 100 RH.

TABLE 6. Mean disease severity rating (MDSR) of two cultivars inoculated with three isolates of *S. trifoliorum* and incubated at four incubation periods in the dew chamber at 18 C and 100% RH. Experiment 1.

TREATMENT			DAYS AFTER INOCULATION MDSR[y]		
CV	INCUBATION TIME	ISOLATE	5	10	15
Arc	24 hr	LAL 3	1.4 a[z]	2.0 a	2.3 a
		CAL 3	1.2 a	1.7 a	2.6 a
		TAL 4	1.1 a	1.8 a	2.2 a
Vertus		LAL 3	1.2 a	1.8 a	2.0 a
		CAL 3	1.0 a	1.7 a	2.1 a
		TAL 4	1.0 a	1.7 a	2.0 a
Arc	48 hr	LAL 3	1.8 a	2.3 a	2.6 a
		CAL 3	1.4 a	2.1 a	2.8 a
		TAL 4	2.0 a	3.0 a	3.5 a
Vertus		LAL 3	1.5 a	2.0 ab	2.3 a
		CAL 3	1.3 a	1.4 a	2.0 a
		TAL 4	2.1 b	2.2 b	2.5 a
Arc	72 hr	LAL 3	2.6 a	3.2 a	3.8 a
		CAL 3	2.0 a	2.6 a	3.2 a
		TAL 4	2.6 a	2.9 a	3.4 a
Vertus		LAL 3	1.8 a	1.9 a	2.1 a
		CAL 3	1.4 a	2.1 a	2.6 a
		TAL 4	2.1 a	2.5 a	2.6 a
Arc	96 hr	LAL 3	2.5 a	3.0 a	3.3 a
		CAL 3	2.0 a	2.5 a	3.1 a
		TAL 4	2.3 a	3.1 a	4.0 a
Vertus		LAL 3	1.8 a	1.9 a	2.0 a
		CAL 3	1.5 a	2.1 a	2.6 a
		TAL 4	2.0 b	3.1 b	4.2 a

[y] MDSR of 25 plants / cv / isolate / incubation period based on a 1-7 disease severity rating scale.

[z] numbers within columns for each cv at a particular incubation period are not significantly different according to Student-Newman-Keul's test for comparison treatment means ($P \leq .05$). Date of inoculation June 16, 1986.

higher disease severity than the 96 hr incubation (TABLE 7). In both experiments the MDSRs were observed to increase from the 5-day to the 15-day rating periods. In all experiments, the non-inoculated control plants did not exhibit disease symptoms. There was no indication of wilting of the plants due to water stress. The bottom-watering procedure appeared to provide sufficient moisture for continued growth of plants.

The above results confirm the reliability of the mycelial plug inoculation technique under these specific conditions. Isolate TAL 4 was more virulent than LAL 3 or CAL 3, and all three differentiated between Vertus and Arc best at 72 hr incubation. The overall infection obtained with all three isolates on both cvs was, however, significantly higher after 96 hr incubation. The advantages of 96 hr incubation time are i) insurance of good infection and symptom development, and ii) prevention of plants escaping disease.

In the experiment testing the suitability of the respective age groups of the cvs against isolate TAL 4 (Age study 1A) the seedlings used were not as vigorous as they should have been under normal growth due to metalaxyl toxicity; TAL 4 caused severe damage on 4-, 6-, and 7-week-old than on 5-, 8-, or 9-week-old plants of Arc and Vertus after 48 hr incubation (TABLE 8). The MDSR of the 9-week-old plants of Arc and MDSRs of the 8- and 9-week-old plants of Vertus were significantly less than those of the 4-week-old plants. After 96 hr period no significant differences were observed in treatments of both

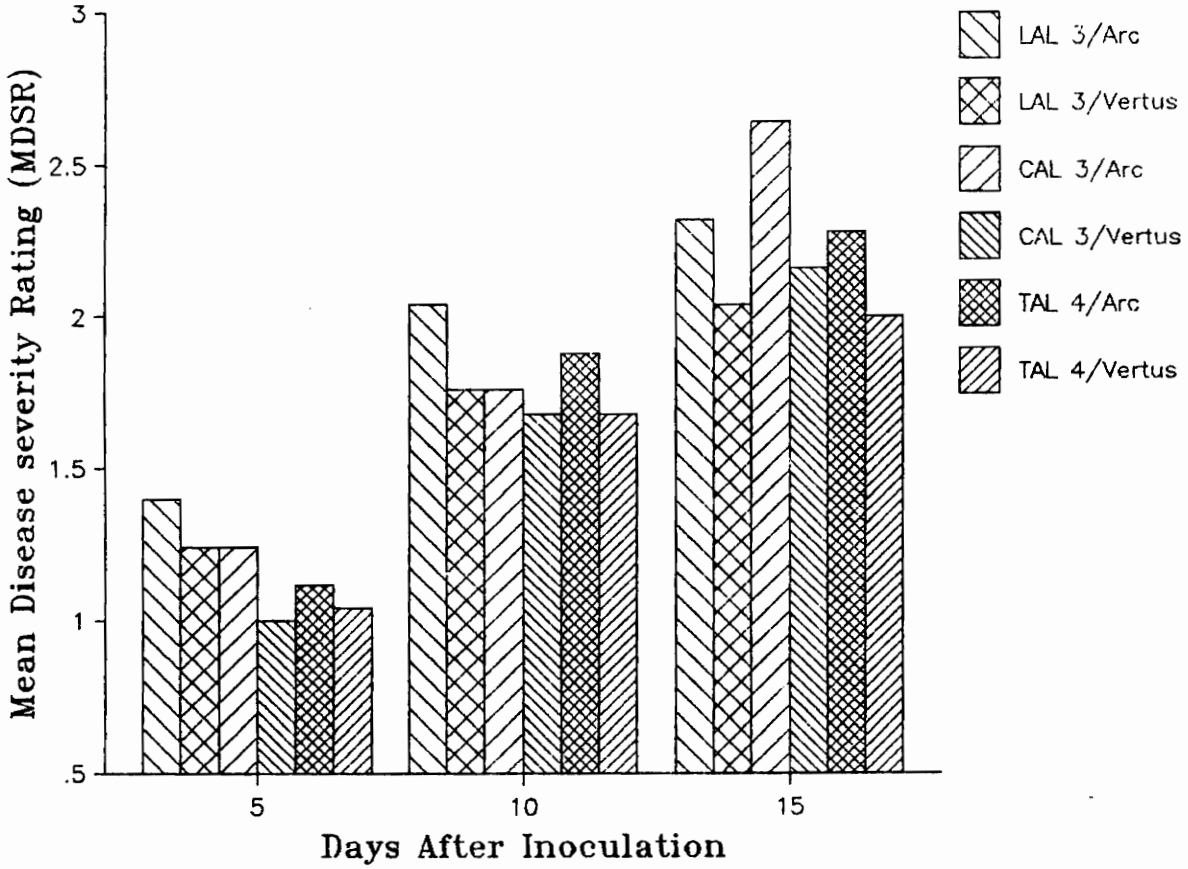

FIG. 4. Influence of 24 hr dew chamber incubation time on disease severity of alfalfa cvs Arc and Vertus inoculated with three isolates of *S. trifoliorum* .

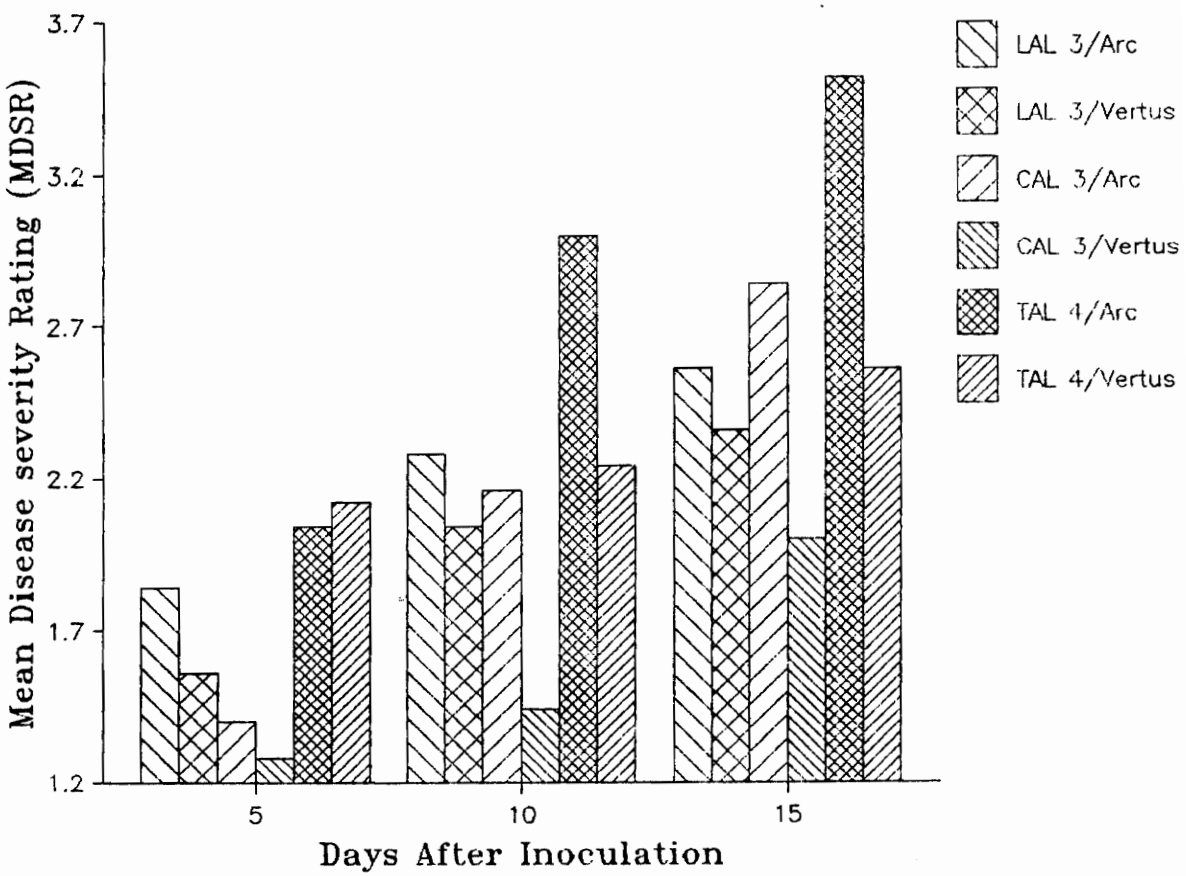

FIG. 5. Influence of 48 hr dew chamber incubation time on disease severity of alfalfa cvs Arc and Vertus inoculated with three isolates of *S. trifoliorum*.

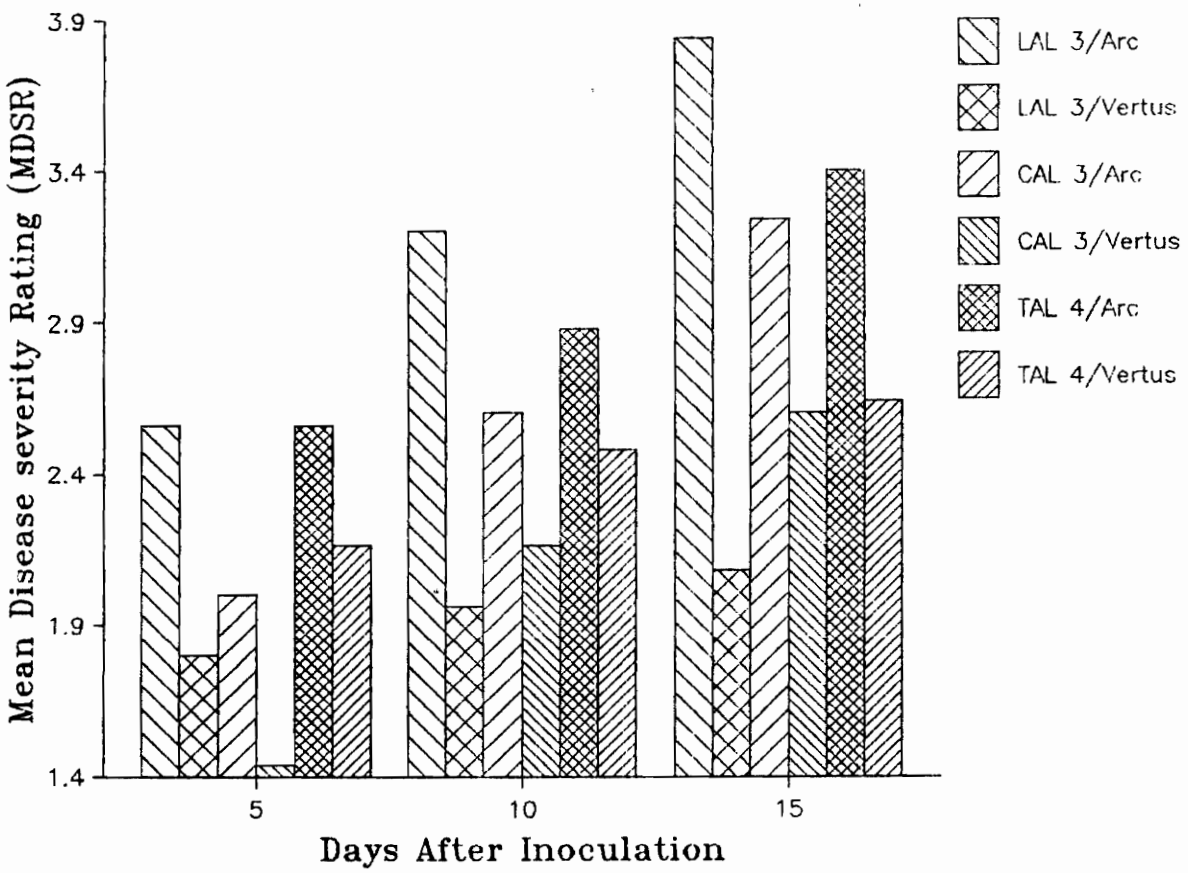

FIG. 6. Influence of 72 hr dew chamber incubation time on disease severity of alfalfa cvs Arc and Vertus inoculated with three isolates of *S. trifoliorum*.

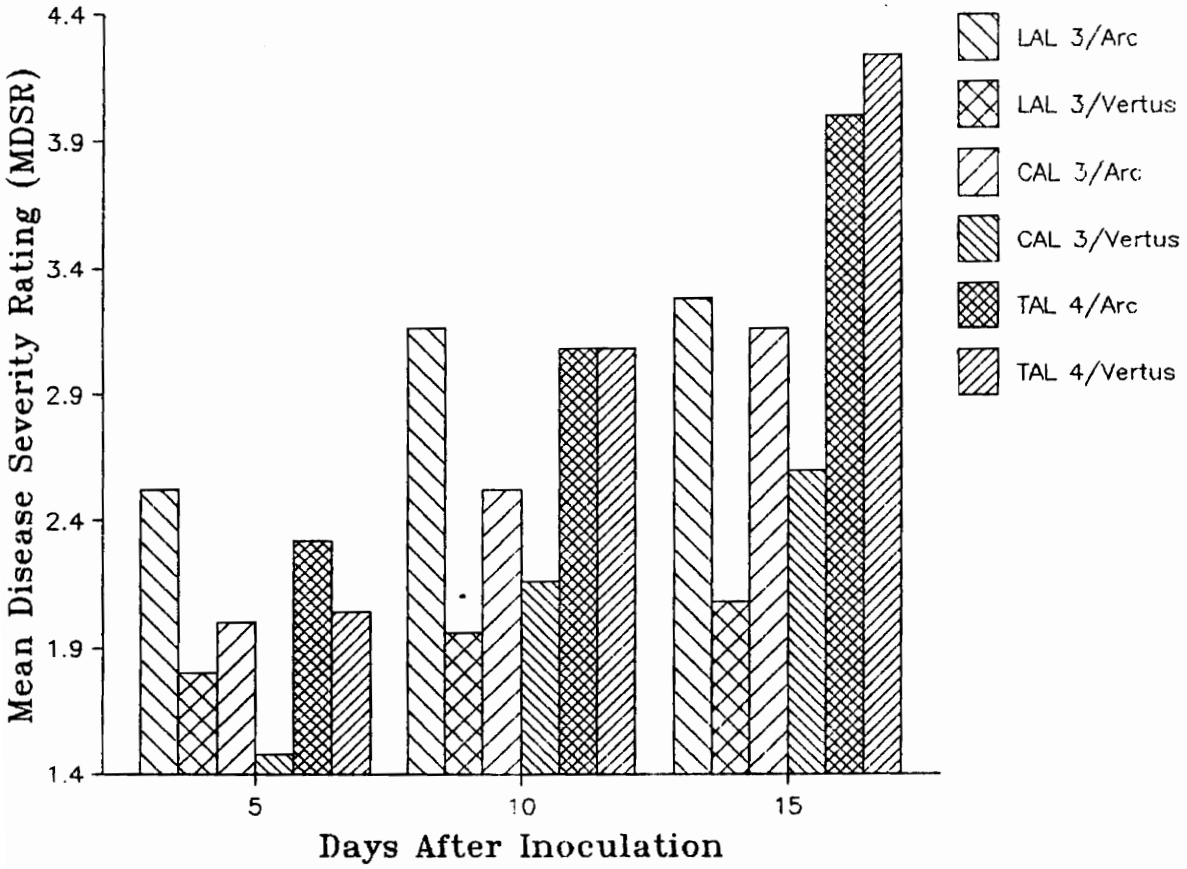

FIG. 7. Influence of 96 hr dew chamber incubation time on disease severity of alfalfa cvs Arc and Vertus inoculated with three isolates of *S. trifoliorum*.

TABLE 7. Mean disease severity rating (MDSR) of two cultivars inoculated with three isolates of *S. trifoliorum* and incubated at four incubation periods in the dew chamber at 18 C and 100% RH. Experiment 2 1986.

TREATMENT			DAYS AFTER INOCULATION MDSR[y]		
CV	INCUBATION TIME	ISOLATE	5	10	15
Arc	24 hr	LAL 3	2.2 a[z]	3.0 a	3.9 a
		CAL 3	1.8 a	2.8 a	3.8 a
		TAL 4	2.2 a	3.5 a	4.4 a
Vertus		LAL 3	2.0 a	2.9 a	3.6 a
		CAL 3	2.0 a	3.2 a	4.1 a
		TAL 4	2.2 a	3.2 a	3.9 a
Arc	48 hr	LAL 3	2.1 a	2.7 a	3.4 a
		CAL 3	2.1 a	2.8 a	3.6 a
		TAL 4	2.7 a	4.3 b	4.8 a
Vertus		LAL 3	2.4 a	3.1 a	4.0 a
		CAL 3	2.6 a	3.4 a	4.0 a
		TAL 4	2.2 a	3.0 a	3.5 a
Arc	72 hr	LAL 3	2.6 a	3.6 a	3.9 a
		CAL 3	2.2 a	3.6 a	4.0 a
		TAL 4	2.5 a	4.1 a	4.7 a
Vertus		LAL 3	2.6 a	3.9 a	4.6 a
		CAL 3	2.9 a	3.4 a	4.0 a
		TAL 4	2.1 a	3.6 a	4.4 a
Arc	96 hr	LAL 3	2.2 a	3.2 a	4.2 a
		CAL 3	2.1 a	3.3 a	3.7 a
		TAL 4	2.7 a	4.3 a	4.7 a
Vertus		LAL 3	1.7 a	2.9 a	3.7 a
		CAL 3	3.0 b	4.0 a	4.7 b
		TAL 4	2.2 ab	3.0 a	3.5 ab

[y] MDSR of 25 plants / cv / isolate / incubation period based 1-7 disease severity rating scale.

[z] numbers within columns for each at a particular incubation period are not significantly different according to Student-Newman-Keul's test for comparison of treatment means ($P \leq .05$). Date of inoculation June 20, 1986.

cvs, however, the MDSRs obtained with the 5-, 8- and 9-weekold plants of both cvs were generally lower. Most of the 4-, 6-, and 7-week-old plants of both cvs were killed within the obser-tion period. Data of age study 1B (repetition of 1A) was not statistically analyzed due to unsatisfactory treatments.

In the second age study, with more mature plants of the two cultivars, the results were interestingly different. The range of disease symptoms produced was low and the intensity of the disease was less than with two-month-old plants. The only symptoms observed were wilting of stems without any stem necrosis and no dead plants. Of the three disease severity ratings taken only the final 15- day data were analysed since the ratings remained without changes on all rating periods. Significant differences were observed in the MDSRs among isolates on both cvs Arc and Vertus, at the two incubation periods; TAL 4 (more virulent on two-month-old plants) was significantly less virulent than either CAL 3 or LAL 3 (TABLE 9). Disease severity was higher after 96 hr incubation than after 48 hr incubation. However, the plants did not develop typical SCSR symptoms and may be highly resistant to infection at this stage of plant growth.

In all of the inoculation tests, a white mycelium growth was observed on the inoculated plants, at and a little above the inoculation site, especially at the time the treatments were removed from the dew chamber. Sclerotia were observed on the dead plants at or slightly below the crown (FIG. 8).

TABLE 8. Mean disease severity rating (MDSR)of six age groups of two alfalfa cvs inoculated with a virulent isolate (TAL 4) of *S. trifoliorum* followed by incubation periods of 48 hr or 96 hr in a dew chamber at 18 C and 100% RH.

CV	Age of Seedling At Inoculation (weeks)					
	4	5	6	7	8	9
48 hr						
ARC	[y]4.8abc[z]	3.4ab	5.2bc	5.5c	3.6ab	3.3a
Vertus	5.5b	4.2ab	4.8ab	4.7ab	3.5a	3.4a
96 hr						
Arc	5.5a	5.0a	5.5a	5.7a	5.2a	4.5a
Vertus	6.0a	5.4a	6.0a	5.0a	4.7a	4.6a

[y] MDSR of 20 plants per cv at 20 days after inoculation on a disease severity rating scale of 1-6.

[z] numbers within a row followed by the same letter are not significantly different according to Student-Newman-Keul's test for comparison of treatment means ($P \leq .05$). Age study 1- date of inoculation July 16, 1986.

TABLE 9. Mean disease severity rating (MDSR) of mature plants (greater than 2-months) of two alfalfa cvs inoculated with three isolates of *S. trifoliorum* followed by 48 hr or 96 hr incubation in a dew chamber at 18 C and 100% RH.

CV[x]	INCUBATION TIME	ISOLATE LAL 3	CAL 3	TAL 4
	48 hr			
Arc		[y]1.1ab[z]	1.3b	1.0a
Vertus		1.3b	1.2b	1.0a
	96 hr			
Arc		1.4b	1.2a	1.2a
Vertus		1.4b	1.3b	1.1a

[x] two-month-old plants were cut back, fertilized and regrown for 2 weeks before inoculation.

[y] MDSR of 50 plants per cv at 15 days after inoculation based on a disease severity rating scale of 1-3.

[z] numbers within a row followed by the same letter are not significantly different according to Student-Newman-Keul's test for comparison of treatment means ($P \leq .05$). Date of inoculation - May 29, 1986.

Pathogenicity of the isolates tested was confirmed by re-isolation of the pathogen from the necrotic region of living plants, and from wilted and dead plants.

DISCUSSION

There were slight differences in the growth rates and the number of sclerotia produced on PDA at 20 C in the dark, among the monoascospore isolates of *S. trifoliorum* tested. All the isolates grew well at this temperature. The failure of these isolates to grow at 28 C but their ability to resume growth on transfer to 20 C indicates that they are sensitive to but not killed at the higher temperature. However, further study is necessary on the physiology, nutrient and temperature requirements of these isolates. The importance of having suitable long term preservation of the stock cultures of these isolates is stressed here in order to prevent the development of abnormal variants. One of the characteristics of *S. trifoliorum* is that when allowed to grow on PDA for several weeks without being transferred, it may undergo an irreversible change after which subculture of this yields a variant that neither forms sclerotia nor causes wilting of clover plants (4). This could happen in the case of these isolates too. However, in the present study, such abnormal variation was not observed in any of the isolates. They continued to produce sclerotia and the colonies resembled the parent cultures. In order to prevent loss of pathogenicity, frequent inoculation on to appropriate hosts and re-isolation of the isolates is essential (5); alternatively, the cultures could

be lyophilized or sclerotia produced in culture can be stored under dry conditions.

The following criteria were used for selection of five isolates from the group of eleven since i) they had slow to moderate growth on PDA at 5 days, and ii) sclerotia were not present, an indication that the colonies were actively growing. Raynal (11) reported that cultures with sclerotia were not pathogenic. It was observed that the isolates had only a trace of growth on the medium after 24 hr and as a result, three days after transfer, the colony dia was not large enough (11), thus 5-day-old cultures, as opposed to 3-day-old cultures used by Raynal (11), were determined to be more suitable for this study and the results of the preliminary experiment confirmed this. Plugs from the margins rather than from the center of the colonies were used in the present study since it was earlier reported (11) that the former produced higher disease severity on clover plants. It was assumed that the 6mm plugs had hyphal fragments of equal age and that a constant inoculum dosage was utilized each time. However, it would be desirable to quantify this inoculum dosage. The agar plug was used as a carrier for inoculum to improve adherence to the site of inoculation (11) and the possibility of interference of the agar was disregarded. The site of inoculation (crown of plant) was effective in producing the required disease symptoms in both cultivars tested. However, since the number of stems per plant differed among and within cultivars, it might be useful to test plants

with 1 plug per stem as Schmidt (12) did when he used cotton pads with mycelium suspension as inocula. From the present study it cannot be confirmed that the dosage of inoculum used per plant was sufficient although it was enough to kill some plants of both cvs and produce desirable SCSR symptoms. Further investigation in this respect is necessary. A disadvantage of this inoculation technique is that plants can be challenged with only one isolate at a time. It would be interesting to investigate the responses of these alfalfa cvs to a mixture of the isolates either by placing plugs of different isolates on different stems or by placing a slurry of mixed isolates on the crown of a plant. However, the variabilty in stem number within a cv will make simultaneous inoculation with different isolates impractical. It will be desirable if these cultivars could be challenged with an ascospore inoculation technique since cv response to SCSR may depend on its reaction to ascospore infection.

There were highly significant differences in the virulence among the 5 isolates tested on both cvs. Favorable results can be obtained with the mycelial plug inoculation technique using any one isolate tested. It was interesting to note that isolate TAL 4 which had faster growth on PDA than LAL 3 was more virulent on two-month-old plants of both cvs while the slower growing isolate LAL 3 which also tends to produce a larger number of sclerotia on PDA but much later than the other isolates tested was less virulent under these experimental

conditions. However, LAL 3 appeared to be more virulent on the more mature plants (greater two-month-old) used in the age study 2. The difference in the pathogenicity of the different isolates on alfalfa cvs are not unexpected since it has been reported that different monoascospore isolates varied distinctly in pathogenicity to Ladino clover (7).

Selection of three isolates (LAL 3, CAL 3, TAL 4) for the repeat experiments was due to the fact that there were no significant differences between the 2 CAL isolates or the 2 TAL isolates in the preliminary study. The three isolates, however, did have a range in pathogenicity. In experiments 1 and 2 it was confirmed that isolate TAL 4 was more virulent on both cvs. Infection and colonization of plants with production of typical symptom development occurred in all four dew chamber incubation periods tested. However, cultivar screening for resistance are best at 72 or 96 hr since they will enable development of higher disease severity among the cvs and severe enough conditions to prevent escapes from disease, without killing all the plants and at the same time allow differences between cultivars. With the exception of leafspots which are the result of ascospore infection, typical symptoms of SCSR observed in alfalfa under natural conditions, such as wilting of stems, stem necrosis, plants turning straw color and death, were produced in all of these tests. The slight differences in MDSRs of cvs Arc and Vertus to the three isolates were detected under these inoculation conditions. Differences in the actual

susceptibility of the 2 cvs to the isolates in nature may not be that distinct. In general, the disease severity of a cultivar was dependent on the isolate and the incubation period; the more virulent the isolate and / or longer the incubation time, the greater was the disease severity. The disease severity of plants also tends to increase from 5 to 15 days after inoculation. Thus, observation of disease severity at longer time intervals may be necessary.

Eight- or 9-week-old plants of both cvs Arc and Vertus are less susceptible to attack by *S. trifoliorum* than 4-, 6-, or 7-week-old plants. Most plants in latter groups were killed within the 20-day observation period. This is not a desirable characteristic when screening for resistance. There was less disease severity in the 5-week-old plants when compared to the 6- and 7-week-old plants since the test plants in the latter group were less vigorous and exhibited greater stress symptoms as a result of metalaxyl toxicity. In general all test plants used in this study lacked vigor and uniformity. Thus, this should be repeated with sets of plants of normal vegetative growth. An observation in the course of this study was that plant vigor is important in determining its ability to withstand infection. It is, thus, essential that all plants be of equal age, vigor and without stress symptoms when used for cultivar evaluation studies.

The failure of mature (greater than 2-month-old) plants to produce distinct disease symptoms and the absence of stem

necrosis and dead plants clearly indicate that they are more resistant to infection by *S. trifoliorum* under these experimental conditions. This may be due to more effective anatomical or physiological barriers of mature plants. Thus the use of plants older than two-months for disease screening studies may not be beneficial. Eight- to nine-week-old plants are the most suitable.

Though a simple disease severity rating scale of 1-3 was used in the preliminary study, a different system was found necessary in the later experiments since there was a range in percentage disease observed among tested plants. A scale of 1-7 was used in experiments 1 and 2 and scale of 1-6 in the age study 1A and 1B. Plants with wilt without any necrosis were not observed in the age study, and thus not considered in the latter. Although 3 scales have been used, the 1-6 scale, developed in this study, was found to be most useful for evaluation of cultivars.

The mycelial plug inoculation technique under controlled temperature and relative humidity conditions with minimum watering after incubation can be successfully used for screening of alfalfa cultivars under greenhouse conditions. Although all three *S. trifoliorum* isolates and both 72 hr and 96 hr dew chamber incubation periods appeared to distinguish between disease responses of cvs Arc and Vertus, it was decided to evaluate cvs under greater disease pressure. Thus, the more virulent isolate TAL 4, the 96 hr incubation period (both of

which produced significantly higher MDSRs on both cvs), 8- 9-week-old plant age and a disease severity rating scale of 1-6 were selected for cultivar screening experiments in the second part of this study under objective 2.

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CHAPTER 3

Evaluation of Resistance to Sclerotinia Crown and Stem Rot Caused by *Sclerotinia trifoliorum* in Selected Alfalfa Cultivars in the Greenhouse.

INTRODUCTION

Sclerotinia crown and stem rot (SCSR) caused by *Sclerotinia trifoliorum* Eriks. is a common and destructive disease on clovers (*Trifolium* spp.) and alfalfa (*Medicago sativa* L.) in temperate regions of the world. In recent years, SCSR has been shown to cause severe stand losses, particularly in fall-seeded, no-tillage alfalfa plantings in Virginia (11, 12, 13). Disease management strategies such as crop rotation with non-susceptible crops, grazing or clipping of alfalfa top growth to allow rapid drying of the soil surface and thus reduce inoculum levels or soil treatment with the fungicide pentachloronitrobenzene (PCNB) have neither proven effective nor economical (8). Other practices such as flooding of fields or deep burial of sclerotia prior to planting are not feasible in fields where no-tillage methods are encouraged. No alfalfa cultivar (cv) resistant to SCSR is currently available and the need for one is is urgent since disease resistant germplasm is a component of integrated disease management of any crop.

Field evaluation of resistance in forage legumes to SCSR is difficult because of the erratic occurrence of the disease from year to year and from plot to plot (6). Raynal (7) developed a

a mycelial plug inoculation technique which enabled him to distinguish significant differences in tolerance levels to SCSR among some clover cvs, a few alfalfa cvs, and some *Medicago* spp. A modification of this technique was found to be reliable in detecting differences between the disease responses of two alfalfa cvs Arc and Vertus as reported in the previous chapter.

Inoculation techniques have also been developed to evaluate alfalfa cvs for resistance to root and crown rot caused by *Fusarium* spp. and to root rot caused by *Phytophthora* sp. out under greenhouse conditions (9). Work has also been conducted on the evaluation of disease resistance of soybean (*Soja* spp.) and bean (*Phaseolus* spp.) cvs to *Sclerotinia sclerotiorum* (2).

The objective of this study was to determine the feasibility of using the mycelial plug inoculation technique to evaluate disease resistance in selected alfalfa cvs under greenhouse conditions. Questions that need to be answered are: i) does this technique give reliable and repeatable results whereby significant differences can be detected among the cvs tested? ii) are there differences in the behavior of these cvs over time during the observation period? 3) what is the best period for rating disease symptoms? 4) is the rating scale adopted to evaluate the cvs an appropriate one? and 5) can this technique be used as a 'sieve' for obtaining more disease resistant germplasm?

Due to its autotetraploid and cross-pollinating nature, alfalfa is a highly heterogeneous crop (5). Thus individual plants within most alfalfa cvs vary for many characteristics including disease resistance. The heterogeneous nature of the alfalfa cv makes selection for disease resistance more difficult than for other more genetically homozygous crops. Phenotypic selection in a heterogeneous population has been the most effective method of breeding for alfalfa disease resistance. Recurrent selection utilizing mass screening of young seedlings and intercrossing 100 or more resistant plants has been effective in the selection of resistance to rust, common leafspot, bacterial wilt, anthracnose, *Fusarium* wilt and *Phytophthora* root rot (5). Any plant that does not develop disease symptoms when inoculated and incubated under optimal conditions may be a potential source of germplasm that could be propagated to obtain breeding lines.

MATERIALS AND METHODS

Twelve alfalfa cultivars (Arc, Vertus, Cimarron, Anstar, Armor, WL 320, Maxim, Saranac AR, Pioneer Brand 526, Euver, Endure and Raidor) were evaluated for disease resistance to a virulent, single, large-ascospore isolate (TAL 4) and a less virulent, single, large-ascospore isolate (LAL 3) of *S. trifoliorum* by a mycelial plug inoculation technique under greenhouse conditions. Two experiments (A & B) in 1986 and three (C, D, E) in 1987 were conducted using the virulent isolate TAL 4 as inoculum with an incubation period of 96 hr after

inoculation in a dew chamber (Percival, model I-60 D, P.O. Box 249, Boone, Iowa 50036) at 18 C and RH approaching 100% and one experiment (F) in 1987, in which the twelve cvs were challenged with LAL 3 under the similar conditions.

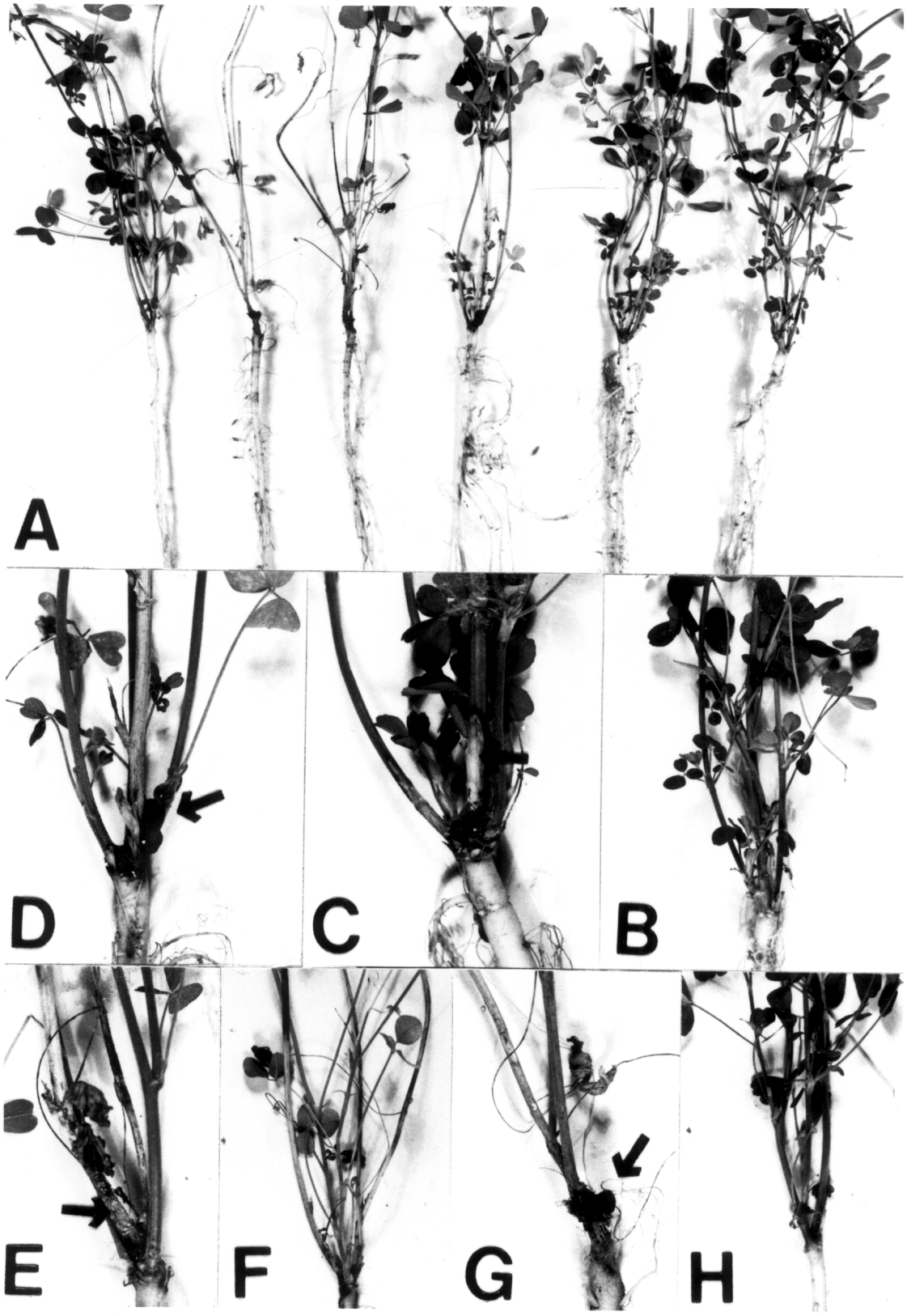
In the 1986 experiments five to six seeds per cv were seeded in Cone-tainers® (Ray Leach Cone-tainer Nursery, Canby, OR 97031) containing a mixture of steam pasteurized soil and peat with vermiculite (1:1:1, potting mix A) that received lime to correct pH to 6.0, *Rhizobium* inoculant (for nodulation) and the fungicide metalaxyl (Apron® Ciba-Geigy) used as a soil drench to prevent damping-off disease caused by *Pythium* spp. Thinning was done at 12 days after planting to one seedling per Cone-tainer®, and a fertilzer treatment in the form of 1 ml per Cone-tainer® of a solution of Na_2HPO_4 (19.6g/l) and KCl (13.1g/l) was applied a day later. At two-months the planting media were brought to saturation prior to inoculation and fifty plants per cv were inoculated individually with 6 mm plugs cut from the margins of 5-day-old vigorously growing cultures of *S. trifoliorum* isolate TAL 4 grown on the same batch of potato dextrose agar (PDA). A single plug was placed on the non-wounded crown area of each plant, near the soil surface and pressed down to touch all stems. For each cultivar two non-inoculated plants served as controls. Plants were completely randomized within trays that were randomly placed in the dew chamber and incubated for 96 hr at 18 C and 100% RH, in the dark. After 96 hr, plants were removed to the greenhouse bench and arranged into the respective

groups. All plants from each cv were maintained on the same bench and trays were moved around on the bench daily to ensure that they received equal amounts of light. In experiment B but not in A, supplemental light provided by high pressure sodium vapor (HPSV) lamp (1000 watt, suspended 4ft. above the plants) was given for 9 hr (3:00 pm -12:00 midnight) daily. In the greenhouse the plants were bottom watered by placing each tray per cv in a larger container with a standard volume of water (26 liters) for 1 minute daily . At no time did the plants receive any top watering after inoculation until the end of the observation period. The plants were rated at 10, 15, and 20 days after inoculation. Disease severity ratings of symptoms for individual plants in all treatments were recorded on a scale of 1 to 6 : 1 = plant with no symptoms (healthy), 2 = plant with stem necrosis and 0-25% stems wilted, 3 = plant with stem necrosis and 26-50% stems wilted, 4 = plant with stem necrosis and 51-75% stems wilted, 5 = plant with stem necrosis and 76-100% stems wilted but alive, and 6 = plant dead). Figure 8 shows the disease symptoms observed in the inoculated plants. The total number of stems per plant was counted to assist the determination of the percentage of stems wilted per plant. Dates of inoculation of experiments A and B were June 30, and July 7, 1986, respectively.

In all three experiments conducted in 1987, (C, D, & E), utilized the same twelve cvs inoculated with isolate TAL 4 using the same experimental design and procedure as before. However,

FIG. 8. A. Range of disease symptoms that were used for evaluation of cvs on a 1-6 disease severity rating scale. From left to right, inoculated but asymptomatic plant (1), plant with stem necrosis (2), plant with 26-50% wilt (3), plant with 76-100% wilt (5), dead plant (6), and non-inoculated control plant.

B. Inoculated but asymptomatic plant (1).
C. Plant showing stem necrosis, 0-25% stem wilt (2)
D. Plant with stem necrosis, 26-50% stem wilt (3). Sclerotia are shown.
*E. Plant with stem necrosis, 51-75% stem wilt (4). Mycelial mat is shown.
F. Plant with stem necrosis and 76-100% stem wilt (5).
G. Plant dead (6). Sclerotia are shown.
H. Non-inoculated control plant.
* not represented in A.


A
D
C
B
E
F
G
H

the test plants were grown in Cone-tainers® containing a soil mixture of steam pastuerized soil with sand, weblite and vermiculite (2:1:1:1, potting mix B), lime, and Rhizobium inoculant. The use of metalaxyl to prevent damping-off was avoided since it was determined that the fungicide caused phytotoxicity symptoms manifested as chlorosis, marginal leaf scorch and stunting which led to abnormal plants for experiments conducted in 1986. The test plants in the 1987 experiments (grown without metalaxyl treatment) appeared without any leaf scorch, taller, and more vigorous. In these experiments also, two-month-old plants were inoculated and incubated under the same temperature and humidity conditions as described earlier. All three experiments received 9 hrs supplemental light provided by HPSV lamps (1000 watt). There was, however, a slight modification in the watering technique. Experiment C of this set (1987) was bottom watered daily for 1 minute as in the previous year's experiments but it was found that the soil surface remained very dry and that the mycelium mat that was formed on the inoculated plants after incubation had dried up which led to poor symptom expression. The disease severity rating of plants did not change between 10 and 20 days after inoculation. It was clear that the moisture regime in and around the site of inoculation (crown area) of plants was not sufficient for further disease development. As a consequence it was necessary to top water the plants of experiments D and E with a fine mist spray nozzle, and increase the duration of

bottom watering time (five minutes daily as opposed to the 1 minute used in the 1986 experiments). A very light misting of the plants was carried out for 4 to 5 days after removal of plants from the dew chamber and care was taken not to drench the plants. This regime provided optimum conditions for disease development and symptom expression. Dates of inoculation for experiments C, D and E were May 11, May 26, and June 1, 1987 respectively.

The same twelve cultivars were evaluated for resistance to the less virulent isolate LAL 3, under the same experimental design and conditions as described for experiments D and E. Experiment F- date of inoculation was June, 16, 1987.

An additional set of the twelve cultivars was also inoculated with an isolate belonging to the SAL group, (SAL 4), which had not been tested in the earlier pathogenicity studies (experiment G). However, only 25 plants were inoculated with 2 non-inoculated controls per cv. The test plants were grown on a soil mixture of steam pasteurized soil with sand, weblite and vermiculite (2:1:1:1, potting mix B) but with metalaxyl treatment to prevent damping-off. These plants exhibited marginal leaf scorch and were of reduced vigor similar to those observed in the 1986 experiments. Date of inoculation was May 5, 1987.

All cv screening experiments conducted in 1987 were rated for disease symptoms on the same 1 to 6 scale as described for experiments A and B at 10, 15 and 20 days after inoculation.

However, experiments D, E and F were also rated at 35 days to determine whether a longer period was necessary to detect any significant differences among cvs.

At the end of 35 days the dead plants of experiment D and E were discarded; plants with no symptoms and those with wilt and necrosis were cut back, fertilized and regrown for 2 to 3 weeks. The total number of stems per plant was recorded. The regrown plants of experiment D with no symptoms were re-inoculated with 5-day-old plugs of isolate TAL 4 and incubated in the dew chamber for 96 hr. The procedure was essentially the same as that described earlier. Date of re-inoculation was July 13, 1987. The plants were rated for disease symptoms on the same 1-6 scale after 10 days. This part of the experiment was conducted as an observation study and thus not statistically analysed.

Disease severity ratings of all experiments inoculated with isolate TAL 4 (A, B ,D & E) were also converted to the simple 1-3 rating scale as proposed by Raynal (7), (1 = plant without symptoms, 2 = plant with wilt / or necrosis, 3 = plant dead), in order to see whether significant responses between cvs can be detected and to determine reliability of the 1-6 rating scale used in the present study. Experiments D and E were also rated on a 1-5 rating system used by Elgin and Beyer (3) for the above mentioned reasons (1 = no infection, 2 = stem lesions, 3 = less than 50% stems dead, 4 = more than 50% stems dead, 5 = plant dead). The respective isolates tested were re-isolated from the

necrotic regions of living plants, wilted stems and dead plants on PDA in all of the cv screening tests.

Suitable analysis of variance (ANOVA) procedures were performed on the disease severity rating scores of all experiments of both years and comparison of treatment means was done by the Student Newman Keul's test (SNK, 15). Arcsin transformation of the percentage wilt was analysed for experiment A (1986).

RESULTS

With the mycelial plug inoculation technique all alfalfa cultivars tested were susceptible to the isolates TAL 4, LAL 3 and SAL 4 under the specific experimental conditions. Sufficient symptom development was observed in all but one of the experiments (C) conducted with isolate TAL 4, and those with isolates LAL 3 and SAL 4, respectively. Re-isolation of the fungus from the infected and dead plants of different cvs on PDA indicates their pathogenicity on the twelve cvs under these conditions.

Slightly significant differences among the mean disease severity ratings (MDSRs) of cvs were determined at the 5% level ($P \leq .05$) in three of the four experiments with TAL 4 (excluding experiment C) and with LAL 3 and SAL 4, respectively. The disease pressure, as revealed by the wider range in the MDSRs (TABLE 10), and the lower percentage of inoculated healthy or asymptomatic plants (TABLE 11), was higher in both experiments A & B of (1986), with isolate TAL 4 than in D and E (1987).

TABLE 10. Mean disease severity rating (MDSR) of twelve, two-month-old greenhouse grown alfalfa cvs inoculated with five-day-old culture of *Sclerotinia trifoliorum* (isolate TAL 4) and incubated for 96 hr in the dew chamber at 18 C and 100% RH.

CULTIVAR	POTTING MIX A[v] 1986				POTTING MIX B 1987			
	EXP.A[w]	R[x]	EXP.B	R	EXP.D	R	EXP.E	R
1.Anstar	[y]2.6a[z]	(1)	3.8ab	(7)	1.9a	(2)	1.6ab	(3)
2.Arc	3.6a	(10)	3.4ab	(2)	2.0a	(3)	1.6ab	(3)
3.Armor	3.1a	(6)	4.3ab	(11)	1.8a	(1)	2.0ab	(9)
4.Cimarron	2.8a	(3)	3.6ab	(6)	2.1ab	(7)	1.6ab	(3)
5.Endure	2.9a	(4)	3.5ab	(5)	2.1ab	(7)	1.5a	(1)
6.Euver	3.8a	(12)	3.9ab	(10)	2.0a	(3)	1.5a	(1)
7.Maxim	3.5a	(9)	3.8ab	(7)	2.1ab	(6)	2.0ab	(9)
8.Pioneer526	3.1a	(6)	4.5b	(12)	2.9a	(12)	2.2ab	(11)
9.Raidor	3.7a	(11)	3.8ab	(7)	2.6ab	(11)	2.3b	(12)
10.Saranac AR	3.0a	(5)	3.4ab	(2)	2.5ab	(10)	1.6ab	(3)
11.Vertus	3.1a	(6)	2.9a	(1)	2.0ab	(3)	1.8ab	(8)
12.WL 320	2.7a	(2)	3.4ab	(2)	2.5ab	(9)	1.6ab	(3)

[v]Potting mix A = soil:peat:vermiculite (1:1:1)
Potting mix B = soil:sand:vermiculite:weblite (2:1:1:1)
[w]Four Separate experiments with the same cvs of equal age, inoculated with the same isolate.
[x]Ranking of cv according to ascending order of MDSR.
[y]MDSR of 50 plants per cv on a disease severity scale of 1-6, at 20 days after inoculation.
[z]values within columns followed by the same letter are not significantly different using Student-Newman-Keul's test for comparison of treatment means ($P \leq .05$).

TABLE 11. Percentage of inoculated, non-infected (healthy) plants of twelve alfalfa cultivars at 20 days after inoculation with *Sclerotinia trifoliorum* (isolate TAL 4) and incubated in the dew chamber for 96 hr at 18C and 100% RH.

CULTIVAR	PERCENTAGE OF HEALTHY PLANTS % P.mix A[w] 1986 Exp.A[x] 6/30[y]	Exp B 7/7	P.mixB 1987 Exp.D 5/26	Exp. E 6/1
Anstar	40[z]	20	56	62
Arc	20	32	46	60
Armor	16	12	50	52
Cimarron	28	16	34	60
Endure	20	14	52	66
Euver	22	20	40	58
Maxim	16	18	44	48
Pioneer 526	26	12	24	48
Raidor	20	22	36	40
Saranac AR	40	20	42	66
Vertus	30	36	56	50
WL 320	22	22	40	54

[w] Potting mix A = soil:peat:vermiculite(1:1:1)
Potting mix B = soil:sand:vermiculite:weblite (2:1:1:1)

[x] four separate experiments in which 50 plants of the same 12 cvs were inoculated with the same isolate using a mycelial plug inoculation technique.

[y] Dates of inoculation.

[z] percentage of non-infected plants at 20 days after inoculation.

Figures 9 and 10 show the percentage of inoculated but symptomless plants (apparently healthy), at 20 days after inoculation in the four experiments. The higher disease pressure may be due to either i) greater water holding capacity of the potting mix A used in 1986 (TABLE 12) or ii) stress from metalaxyl toxicity at the time of germination and seedling development. The difference in water holding capacity between the two types of potting mix used in the two years resulted in having to make slight modifications in the method of watering in the greenhouse. Bottom watering of plants for one minute in experiments A and B (1986) grown in potting mix A provided sufficient moisture for the progress of disease and desirable symptom expression on the inoculated plants. The soil surface in the Cone-tainers® was continuously moist. However, this was not true in the 1987 experiments in which plants were grown in potting mix B. This was clearly seen in experiment C in which, though significant differences in cv responses to TAL 4 were obtained at 20 days after inoculation, the only symptoms observed were stem necrosis with less than 2% wilted plants in all cvs, with no dead plant and a very dry soil surface. The symptom expression was comparatively poor, and the range in MDSR of plants was narrow and remained without any changes between the 10-day and 20-day observations (TABLE 13). These are undesirable characteristics in cultivar evaluation studies. In general, the inoculated plants although colonized, appeared healthy and were not much different from a set of non-inoculated

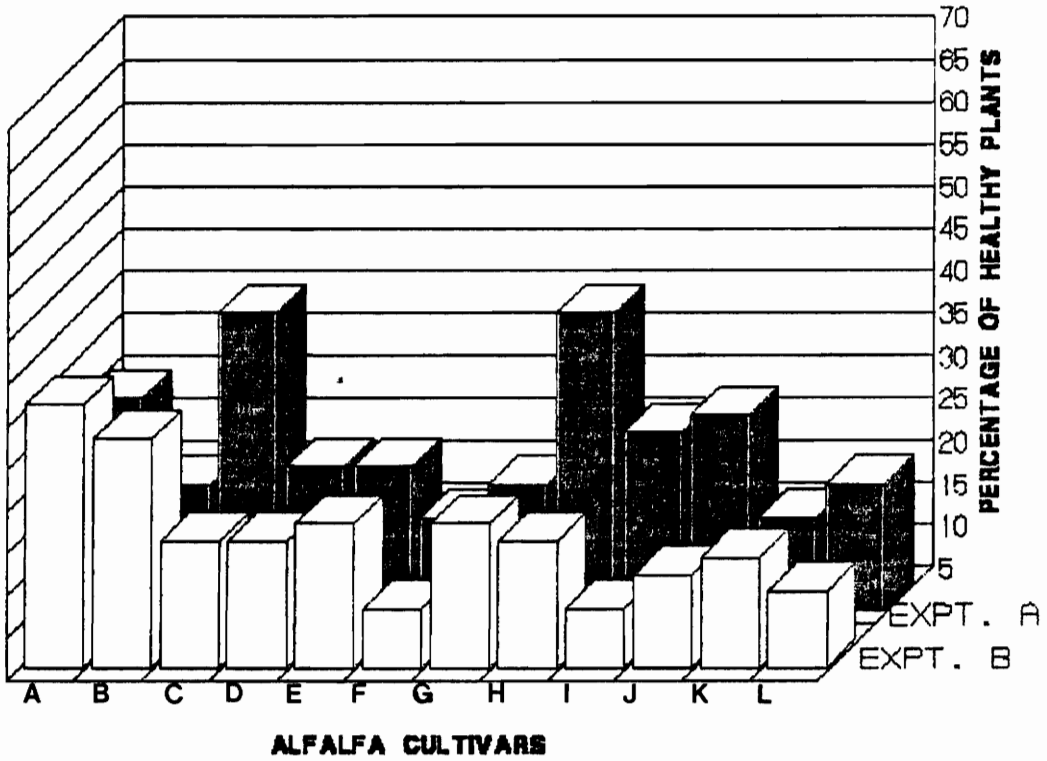


FIG. 9. Percentage of plants of 12 alfalfa cvs that showed no disease symptoms (apparently healthy) at 20 days after inoculation with *S. trifoliorum* (isolate TAL 4), in the two experiments conducted in 1986. A = vertus, B = Arc, C = Saranac AR, D = Euver, E = WL 320, F = Armor, G = Raidor, H = Anstar, I = Pioneer 526, J = Cimarron, K = Maxim, L = Endure. Potting mix A.

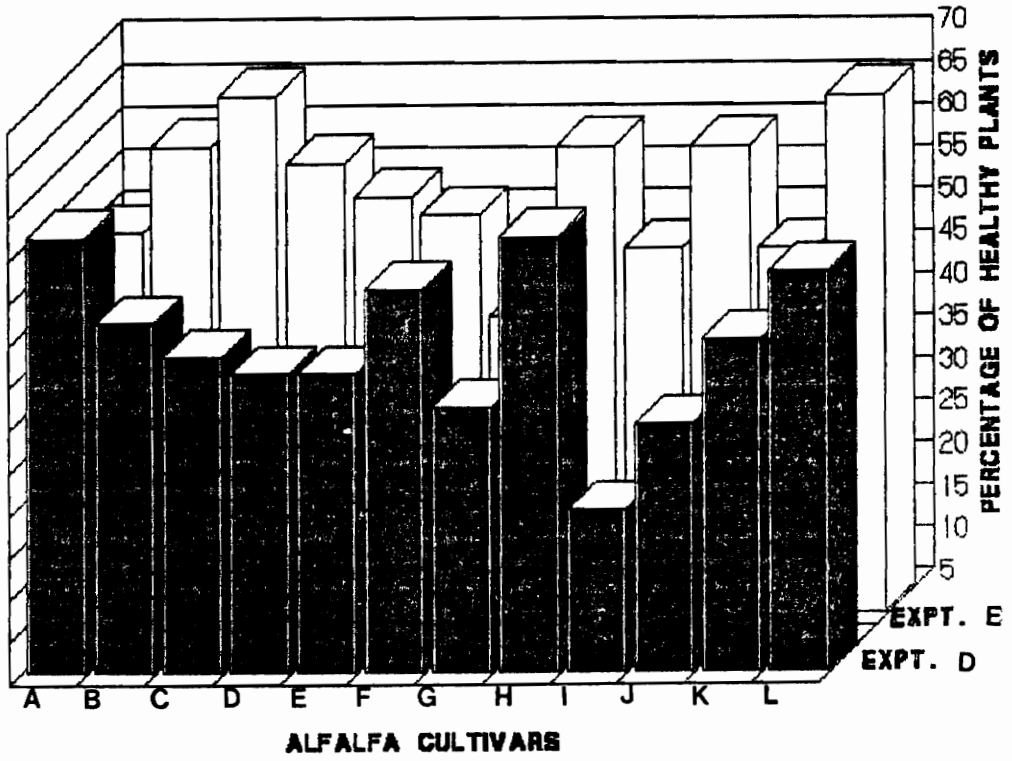


FIG.10. Percentage of plants of 12 alfalfa cvs that showed no disease symptoms (apparently healthy) at 20 days after inoculation with *S. trifoliorum* (isolate TAL 4), in the two experiments conducted in 1987. A = Vertus, B = Arc, C = Saranac AR, D = Euver, E = WL 320, F = Armor, G = Raidor, H = Anstar, I = Pioneer 526, J = Cimarron, K = Maxim, L = Endure. Potting mix B.

TABLE 12. Water holding capacity data of the two potting mixes used in the greenhouse disease screening studies.

PRESSURE (BARS)	WATER HOLDING CAPACITY (AVG.%)	
	[a]Potting mix A	[b]Potting mix B
0.1	36.79	22.16
0.3	33.58	17.23
1.0	30.98	13.50
15.0	10.00	4.57

[a]potting mix A used in 1986 experiments. (soil:peat:vermiculite, 1:1:1)
[b]potting mix B used in 1987 experiments. (soil:sand:vermiculite:weblite, 2:1:1:1).

plants. The use of 1-6 rating scale was not fully justified because there were only a few plants in the 3-5 category (greater than 25% stems / plant wilted) and none with a score of 6 i.e. dead plant. This may be due to a combination of factors such as i) the lack of sufficient moisture and ii) high average daily temperature (22 C-26 C) in the greenhouse, for continued colonization and development of the disease with proper symptom expression in the infected plants. The mycelium mat present on the stems and crown area of plants dried out within a day after removal from the dew chamber; there was no sign of further growth. As a consequence it was decided that all further experiments utilizing potting mix B should be top watered very lightly with a fine mist spray nozzle without drenching plants. Experiments D, E (with TAL 4) and F (with LAL 3) produced sufficient symptom expression to permit the use of 1-6 disease severity rating scale with success.

The reactions of the various alfalfa cvs to isolate TAL 4 were observed to be different in the four experiments in both years, as seen in the changes in the rankings of cvs from experiment to experiment (TABLE 10). Cultivar Anstar, however, had the most consistent tolerance to the pathogen, in that it always appeared higher in the rankings, with comparatively lower MDSRs in three experiments (TABLE 10). Even in experiment B in which Anstar had a fairly high MDSR and ranked 7th, about 20% of the inoculated plants either escaped infection or were highly resistant. Overall, Anstar had greater than 40% asymptomatic

TABLE 13. Mean disease severity rating (MDSR) of twelve cultivars of alfalfa inoculated with five-day-old culture of *S. trifoliorum* (isolate TAL 4) and incubated for 96 hr at 18C and 100% RH. (Exp.C). Date of inoculation-5/11/1987.

Cultivar[y]	[x]MDSR DAYS AFTER INOCULATION 10	20
1. Anstar	1.3ab[z]	1.3ab
2. Arc	1.4ab	1.4ab
3. Armor	1.3ab	1.3ab
4. Cimarron	1.3ab	1.3ab
5. Endure	1.3ab	1.3ab
6. Euver	1.3ab	1.3ab
7. Maxim	1.2ab	1.2ab
8. Pioneer 526	1.2ab	1.2ab
9. Raidor	1.3ab	1.3ab
10. Saranac AR	1.2a	1.2a
11. Vertus	1.6b	1.6b
12. WL 320	1.4ab	1.4ab

[x]MDSR of fifty plants per cv on a disease severity rating scale of 1-6.

[y]cultivars grown in potting mix B, soil:sand:weblite : vermiculite (2:1:1:1). Plants were two-month-old at inoculation and were bottom watered only after removal from the dew chamber. Daily average temperature in the greenhouse was between 22 C-26 C.
Ref. Appendix A.

[z]numbers within a column followed by the same letter are not significantly different according to Student-Newman-Keul's test for means separation ($P \leq .05$).

plants in three of the four experiments (TABLE 11). Cultivars WL 320, and Vertus, also had generally good tolerance to TAL 4 over the four experiments with respect to the MDSRs and their respective rankings (TABLE 10). Cultivars Pioneer Brand 526 and Raidor were found to be the most susceptible in majority of the four experiments under these conditions (TABLE 10). Although cv Saranac AR did not have very low MDSRs in two of the four experiments (TABLE 10), it had 40% or greater percentage of asymptomatic plants in three experiments (TABLE 11).

Considering Experiments A and B (1986, potting mix A), besides the above mentioned tolerant cvs, Endure, Arc and Cimarron, were also fairly tolerant while Euver and Maxim were rather susceptible (TABLES 14, 15). Cultivar Vertus was significantly less susceptible than Pioneer Brand 526 in experiment B while no significant differences were observed in experiment A (TABLES 14, 15). Figures 11 and 12 show the progress of disease in the above two experiments from 10 to 20 days after inoculation. Analysis of the arcsin transformation of percentage wilt of experiment A (1986) revealed that WL 320 was significantly less diseased than Euver. However, there were no major differences in cv rankings between transformed and non-transformed data (TABLE 16).

In experiments D and E of 1987 with TAL 4, additional cvs which exhibited fairly high tolerance were Euver, Endure and Arc (TABLES 17, 18). In experiment D cvs Anstar, Arc, Armor, and Euver were significantly less susceptible than Pioneer

TABLE 14. Mean disease severity rating (MDSR) of twelve cultivars of alfalfa inoculated with five-day old culture of *S. trifoliorum* (isolate TAL 4) followed by 96 hr incubation at 18 C and 100% RH. Ex. A 1986. Date of inoculation 6/30/1986.

Cultivar[x]	[w]MDSR Days After Inoculation 10	15	20	R[y]
1. Anstar	2.3a[z]	2.6a	2.6a	1
2. Arc	3.3a	3.5a	3.6a	10
3. Armor	2.8a	2.9a	3.1a	6
4. Cimarron	2.5a	2.8a	2.8a	3
5. Endure	2.8a	2.8a	2.9a	4
6. Euver	3.4a	3.7a	3.8a	12
7. Maxim	3.3a	3.4a	3.5a	9
8. Pioneer 526	2.8a	3.0a	3.1a	6
9. Raidor	3.2a	3.5a	3.7a	11
10. Saranac AR	2.7a	3.0a	3.0a	5
11. Vertus	2.8a	2.9a	3.1a	6
12. WL 320	2.4a	2.6a	2.7a	2

[w]MDSR of fifty plants per cv rated on a disease severity rating scale of 1-6.

[x]all cultivars were grown in potting mix A, soil:peat: vermiculite (1:1:1). Plants were 2-month-old at inoculation.

[y]Ranking of cv according to ascending order of MDSR.

[z]numbers within a column followed by the same letter are not significantly different according to Student-Newman-Keul's test for mean separation ($P \leq .05$).

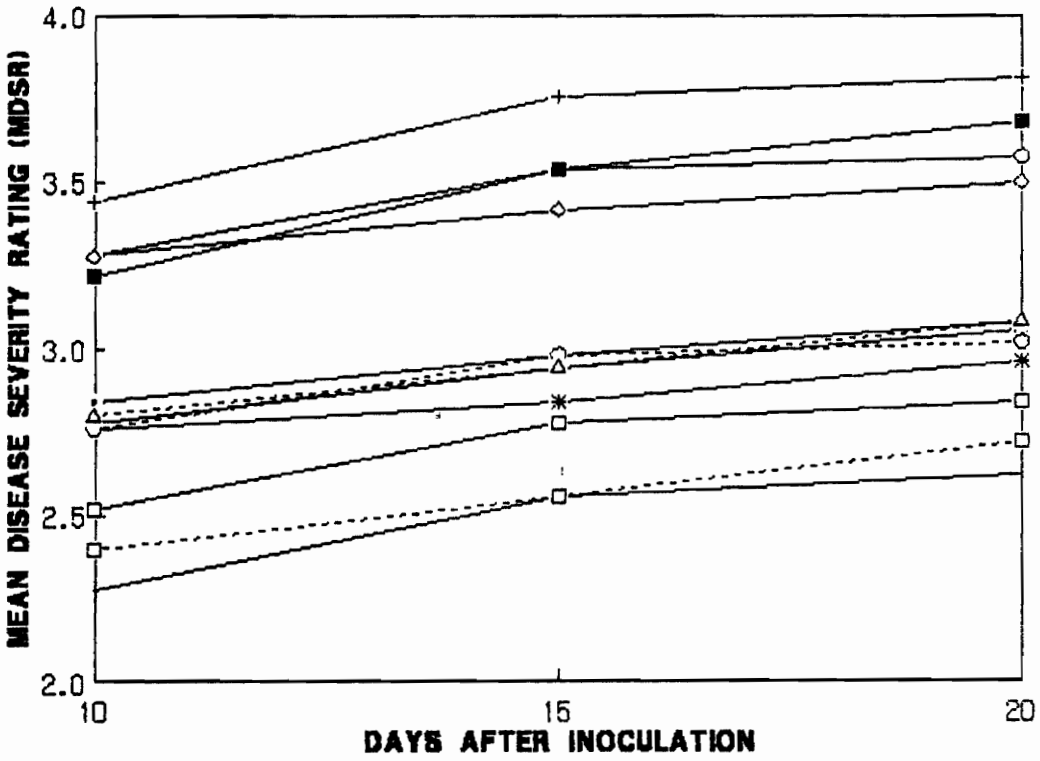


FIG.11. Progress (increase) of disease severity of 12 alfalfa cvs inoculated with *S. trifoliorum* (isolate TAL 4) between 10 and 20 days after inoculation. Anstar =—, Arc =—o—, Armor =—Δ—, Cimarron =—□—, Endure =—*—, Euver =—+—, Maxim =—◇—, Raidor =—■—, Pioneer =-•—•—, Saranac =--○--, Vertus =—-Δ--, WL 320 =--□--. (Exp.A 1986- potting mix A).

TABLE 15. Mean disease severity rating (MDSR) of twelve cultivars of alfalfa inoculated with five-day-old culture of *S. trifoliorum* (isolate TAL 4) followed by 96 hr incubation at 18 C and 100% RH. Exp. B 1986. Date of inoculation-7/7/1986.

Cultivar[x]	[w]MDSR Days After Inoculation 10	15	20	R[y]
1. Anstar	3.3 ab[z]	3.6 ab	3.8 ab	7
2. Arc	2.8 ab	3.3 a	3.4 ab	2
3. Armor	3.3 ab	3.9 ab	4.3 ab	11
4. Cimarron	3.0 ab	3.4 ab	3.6 ab	6
5. Endure	2.9 ab	3.2 a	3.5 ab	5
6. Euver	3.1 ab	3.5 ab	3.9 ab	10
7. Maxim	3.2 ab	3.6 ab	3.8 ab	7
8. Pioneer 526	3.9 b	4.5 b	4.5 b	12
9. Raidor	3.0 ab	3.6 ab	3.8 ab	7
10. Saranac AR	2.9 ab	3.3 a	3.4 ab	2
11. Vertus	2.3 a	2.7 a	2.9 a	1
12. WL 320	2.8 ab	3.2 a	3.4 ab	2

[w]MDSR of fifty plants per cv on a disease severity rating scale of 1-6.

[x]all cultivars were grown in potting mix A, soil:peat: vermiculite (1:1:1). Plants were 2 month-old at inoculation.

[y]Ranking of cv according to ascending order of MDSR.

[z]numbers within a column followed by the same letter are not significantly different according to Student-Newman-Keul's test for mean separation ($P \leq .05$).

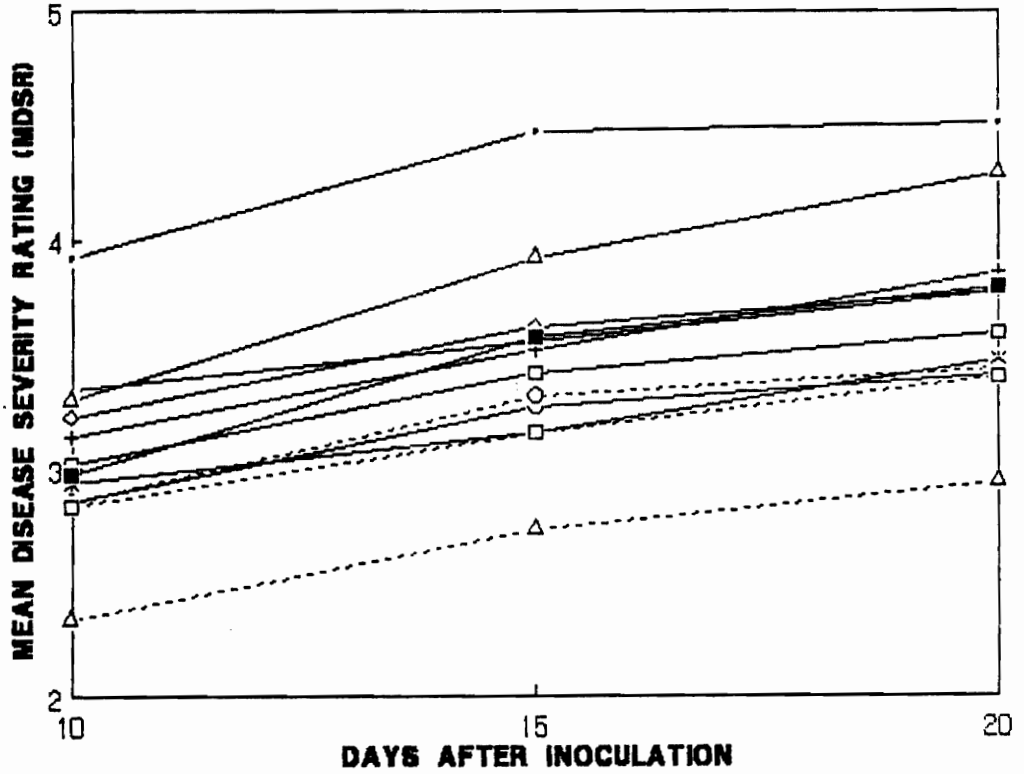


FIG.12. Progress (increase) of disease severity of 12 alfalfa cvs inoculated with *S. trifoliorum* (isolate TAL 4) between 10 and 20 days after inoculation. Anstar =—•—, Arc =—○—, Armor =—Δ—, Cimarron =—□—, Endure =—✳—, Euver =—+—, Maxim =—◊—, Pioneer =—•—•—, Raidor =—■—, Saranac =--○--, Vertus =--Δ--, WL 320 =-·□·-.
(Exp. B 1986-potting mix A).

TABLE 16. Arcsin transformation of the percent wilt data of experiment A (86) of twelve cultivars inoculated with five-day-old culture of *S. trifoliorum* (isolate TAL 4) followed by 96 hr incubation at 18 C and 100% RH.

Cultivar	% wilt[w]	R^x	R^y
1. Anstar	26.1 ab[z]	2	1
2. Arc	42.6 ab	10	10
3. Armor	31.7 ab	5	6
4. Cimarron	28.9 ab	3	3
5. Endure	30.5 ab	4	4
6. Euver	48.4 b	12	12
7. Maxim	40.5 ab	9	9
8. Pioneer 526	33.4 ab	6	6
9. Raidor	42.8 ab	11	11
10. Saranac AR	33.7 ab	7	5
11. Vertus	34.0 ab	8	6
12. WL 320	24.2 a	1	2

[w]arcsin transformation was performed on percent wilt of fifty plants per cv at 20 days after inoculation.

[x]Ranking of cv according to ascending order of transformed data.

[y]Ranking of cv according to ascending order of Mean Disease Severity Rating (MDSR).

[z]numbers within a column followed by the same letter are not significantly different according to Student Newman-Keul's test for mean separation ($P \leq .05$).

Brand 526 (TABLE 17, FIG. 13); while in experiment E Endure and Euver were significantly less susceptible than Raidor (TABLE 18, FIG. 14).

When the disease severity symptoms of the above mentioned four experiments were rated on the simple 1-3 scale of Raynal (7), only minor differences in rankings were found. However, the significant differences detected with the 1-6 rating scale were not observed in two of the four experiments (TABLE 19). In contrast to the 1-6 rating scale, no significant differences were observed in experiment E (1987) when converted to the 1-5 rating scale. No major differences were found between the results of experiment D (1987) using both rating scales. With the 1-5 rating scale, however, cv Anstar had the least MDSRs and ranked number 1 in both experiments (TABLE 20)in contrast to rankings 2 and 3 with the 1-6 scale (TABLE 17, 18). Thus the 1-6 disease rating scale, based on the percentage wilt of individual plants appears to be more sensitive and as a result more useful and reliable in detecting significant differences among cvs than 1-3 or 1-5 scales.

Although symptom development was similar, cultivar rankings obtained with isolate LAL 3 (experiment F) were slightly different from those found with TAL 4 (C.f. TABLES 10 and TABLE 21). Cultivars Euver and Armor were significantly less diseased than Saranac AR, on all three observation dates while WL 320, and Anstar were also highly tolerant (Anstar was ranked 4^{th}). Cultivar Vertus, Maxim, Endure and Arc were moderately

TABLE 17. Mean disease severity rating (MDSR) of twelve cultivars of alfalfa inoculated with five-day-old culture (PDA) of *S. trifoliorum* (isolate TAL 4) and incubated for 96 hr at 18 C and 100% RH. Exp. D. Date of inoculation 5/26/1987.

Cultivar[x]	[w]MDSR Days After Inoculation				R[y]
	10	15	20	35	
1. Anstar	1.6a[z]	1.7a	1.9a	1.9a	2
2. Arc	1.5a	1.8a	2.0a	2.0a	3
3. Armor	1.5a	1.6a	1.8a	1.8a	1
4. Cimarron	1.6a	2.0ab	2.1ab	2.1ab	6
5. Endure	1.8ab	2.0ab	2.1ab	2.2ab	8
6. Euver	1.6a	1.8a	2.0a	2.0a	3
7. Maxim	1.8ab	2.0ab	2.1ab	2.1ab	6
8. Pioneer 526	2.4b	2.7b	2.9b	2.9b	12
9. Raidor	1.9ab	2.4ab	2.6ab	2.6ab	10
10. Saranac AR	2.2ab	2.4ab	2.5ab	2.6ab	10
11. Vertus	1.6a	1.8a	2.0ab	2.0ab	3
12. WL 320	2.1ab	2.4ab	2.5ab	2.5ab	9

[w]MDSR of fifty plants per cv on disease severity rating scale of 1-6. No changes or very slight changes observed between 20 and 35 days observation.

[x]all cultivars were grown in potting mix B soil:sand: weblite: vermiculite (2:1:1:1). Plants were 2-month-old at inoculation.

[y]Ranking of cv according to ascending order of MDSR(35 Da).

[z]numbers within a column followed by the same letter are not significantly different according to Student-Newman-Keul's test for mean separation ($P \leq .05$).

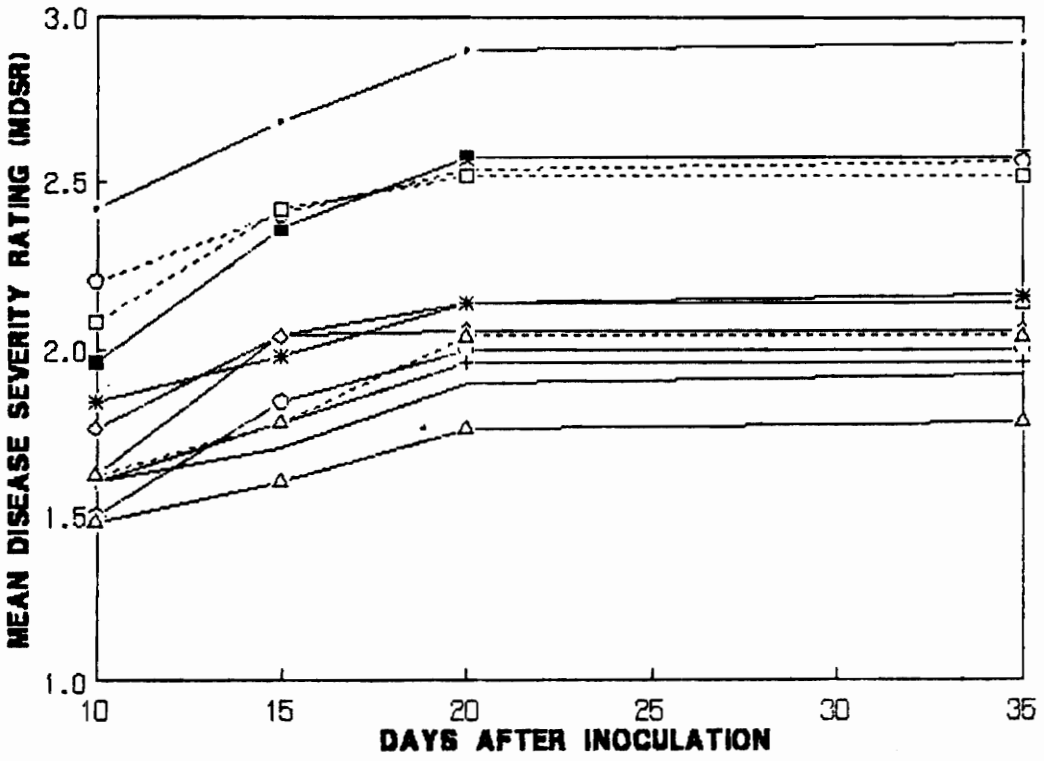


FIG.13. Progress of disease severity of 12 alfalfa cvs between 10 and 35 days after inoculation with *S. trifoliorum* (isolate TAL 4). Anstar =—, Arc =—○—, Armor =—△—, Cimarron =—□—, Endure =—✳—, Euver =—+—, Maxim =—◇—, Pioneer =—•—, Raidor =—■—, Saranac =--○--, Vertus =--△--, WL 320 =--□---. (Exp.D 1987-pottting mix B).

TABLE 18. Mean disease severity rating (MDSR) of twelve cultivars of alfalfa inoculated with five-day-old culture of *S. trifoliorum* (isolate TAL 4) and incubated for 96 hr at 18 C and 100% RH. Exp. E. Date of inoculation-6/1/1987.

Cultivar[x]	[w] MDSR Days After Inoculation 10	15	20	35	R[y]
1. Anstar	1.4a[z]	1.5a	1.6ab	1.6ab	3
2. Arc	1.5ab	1.5a	1.6ab	1.6ab	3
3. Armor	1.9ab	1.9ab	2.0ab	2.0ab	9
4. Cimarron	1.4ab	1.6ab	1.6ab	1.6ab	3
5. Endure	1.4ab	1.5a	1.5a	1.5a	1
6. Euver	1.4a	1.4a	1.5a	1.5a	1
7. Maxim	1.9ab	2.0ab	2.0ab	2.0ab	9
8. Pioneer 526	2.0b	2.2b	2.2ab	2.2ab	11
9. Raidor	2.0a	2.2b	2.3b	2.4b	12
10. Saranac AR	1.4ab	1.5ab	1.6ab	1.6ab	3
11. Vertus	1.5ab	1.7ab	1.8ab	1.8ab	8
12. WL 320	1.4ab	1.5a	1.6ab	1.6ab	3

[w]MDSR of fifty plants per cv on a disease severity rating scale of 1-6.

[x]all cultivars were grown in potting mix B, soil:sand: weblite:vermiculite (2:1:1:1). Plants were 2-month-old at inoculation.

[y]Ranking of cv according to ascending order of MDSR (35da).

[z]numbers within a column followed by the same letter are not significantly different according to Student-Newman Keul's test for mean separation ($P \leq .05$).

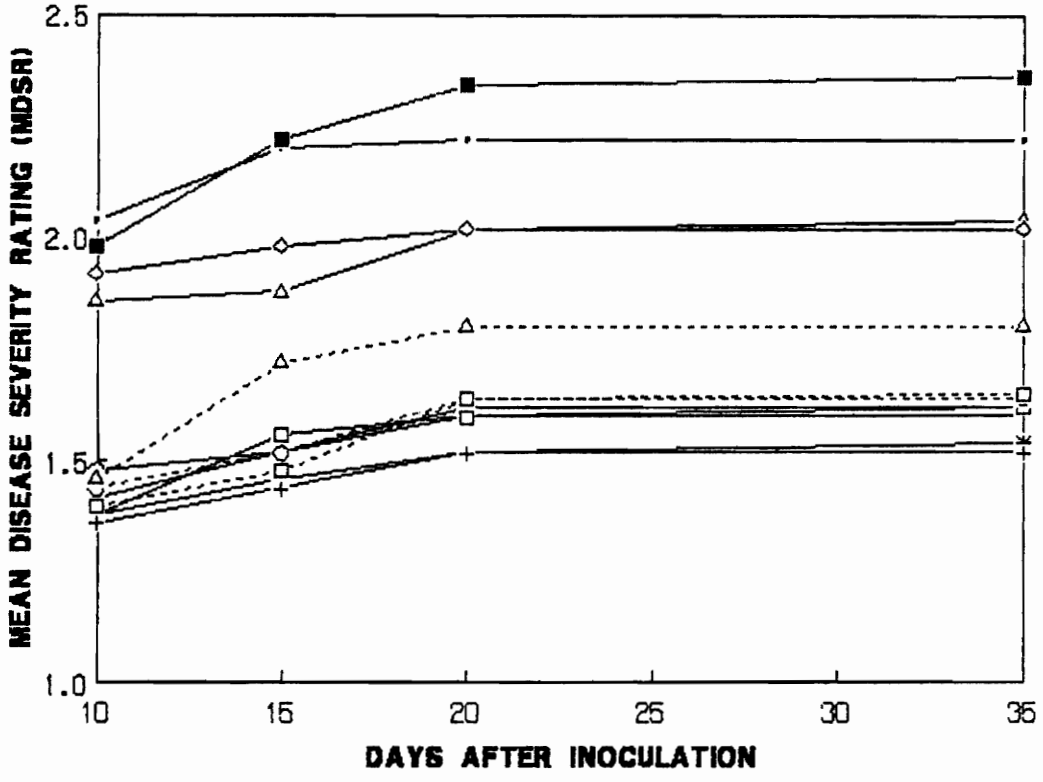


FIG.14. Progress in disease severity of 12 alfalfa cvs between 10 and 35 days after inoculation with *S. trifoliorum* (isolate TAL 4).
Anstar = —, Arc = —O—, Armor = —Δ—,
Cimarron = —□—, Endure = —✳—, Euver = —+—,
Maxim = —◇—, Pioneer = -o—o-, Raidor = —■—,
Saranac = --O--, Vertus = --Δ—, WL 320 = —□---.
(Exp.E 1987-potting mix B).

TABLE 19. Mean disease severity rating (MDSR) on a scale of 1-3, of 12 alfalfa cultivars inoculated with a isolate (TAL 4) of *Sclerotinia trifoliorum* and incubated for 96 hr at 18 C and 100% RH.

Cultivar	[v]MDSR at 20 Days After Inoculation							
	P.mix A[w] 1986				P.mix B 1987			
	[x]Exp.A	[y]R	Exp.B	R	Exp.D	R	Exp.E	R
Anstar	1.8a[z]	1	2.2ab	6	1.5a	1	1.4a	2
Arc	2.0a	5	2.0ab	2	1.6ab	5	1.4a	2
Armor	2.0a	5	2.4b	11	1.5a	1	1.6a	9
Cimarron	1.9a	3	2.2ab	6	1.7ab	7	1.4a	2
Endure	2.0a	5	2.1ab	5	1.5a	1	1.3a	1
Euver	2.1a	10	2.2ab	6	1.7ab	7	1.4a	2
Maxim	2.1a	10	2.2ab	6	1.6ab	5	1.6a	9
Pioneer 526	2.0a	5	2.5b	12	1.9b	12	1.7a	11
Raidor	2.1a	10	2.2ab	6	1.7ab	7	1.7a	11
Saranac AR	1.8a	1	2.0ab	2	1.7ab	7	1.4a	2
Vertus	2.0a	5	1.9a	1	1.5a	1	1.5a	7
WL 320	1.9a	3	2.0ab	2	1.7ab	7	1.5a	7

[v]MDSR of 50 plants per cv on a 1-3 rating scale.
[w]Potting mix A soil:peat:vermiculite (1:1:1).
Potting mix B soil:sand:vermiculite:weblite (2:1:1:1).
[x]Four separate experiments in which the same 12 cultivars were inoculated with the same isolate (TAL 4) incubated for 96 hr.
[y]Ranking of cvs. according to ascending order of MDSR. C.f. with TABLE 10 and 20.
[z]numbers within a column followed by the same letter are not significantly different according to Student-Newman-Keuls's test for mean separation ($P \leq .05$).

TABLE 20. Mean disease severity rating (MDSR) on a scale of 1-5, of 12 alfalfa cultivars inoculated with a virulent isolate (TAL 4) of *S. trifoliorum*, and incubated for 96 hr at 18 C and 100% RH.

Cultivar	[w]MDSR at 20 Days After Inoculation 1987			
	[x]Exp.D	R[y]	Exp.E	R
Anstar	1.8a[z]	1	1.6a	1
Arc	2.0a	2	1.7a	5
Armor	2.0a	2	2.0a	9
Cimarron	2.1ab	6	1.7a	5
Endure	2.1ab	6	1.6a	1
Euver	2.0a	2	1.6a	1
Maxim	2.2ab	8	2.1a	10
Pioneer 526	2.8b	12	2.2a	11
Raidor	2.5ab	10	2.3a	12
Saranac AR	2.4ab	9	1.6a	1
Vertus	2.0a	2	1.8a	7
WL 320	2.5ab	10	1.8a	7

[w]MDSR of 50 plants per cv on a disease severity rating scale of 1-5.

[x]Two separate experiments in which the same 12 cultivars were inoculated with the same isolate (TAL 4) and incubated for 96 hr. (potting mix B).

[y]ranking of cv according to ascending order of MDSR.

[z]numbers within a column followed by the same letter are not significantly different according to Student-Newman-Keul's test for mean separation ($P \leq .05$). C.f. with TABLES 10 and 19.

susceptible while besides Saranac AR, Pioneer Brand 526, Cimarron, and Raidor were the most susceptible at 20 and 35 days after inoculation (TABLE 21, FIG. 15).

Although the symptoms produced were the same, and plants were grown in potting mix B which had a lower water holding capacity, the range in MDSRs was wider in experiment G with isolate SAL 4 (TABLE 22). There were no significant differences among the MDSR of cvs at 20 days after inoculation, however, Arc, Saranac AR, Cimarron, Vertus and Armor were most susceptible while, surprisingly, Pioneer Brand 526, WL 320 and Maxim showed fairly high tolerance and Anstar was moderately susceptible (TABLE 22). It should be stressed here that the test plants used in this experiment exhibited stress symptoms such as marginal leaf scorch and stunting at the time of inoculation due to metalaxyl toxicity and may have contributed to the higher range of disease severity ratings as also manifested in experiments A & B of 1986 though the condition was not as severe.

The disease symptom ratings recorded at 35 days after inoculation (Experiments D & E with TAL 4 and F with LAL 3) were the same as those observed at 20 days (FIGS. 13, 14, 15). There was no indication of any new expanded colonization developing or even changes in symptom expression after this date. The only visible change oberved was the death of plants with 100% stems wilted. The disease severity increased between 10 and 20 days after inoculation and then stabilized.

TABLE 21. Mean disease severity rating (MDSR) of twelve cultivars of alfalfa inoculated with five-day-old culture of *S. trifoliorum* (isolate LAL 3) and incubated for 96 hr at 18 C and 100% RH. Exp. F. Date of inoculation June 16, 1987.

Cultivar[x]	[w] MDSR Days After Inoculation 10	15	20	35	R[y]
1.Anstar	1.8ab[z]	1.9abc	2.0ab	2.0ab	4
2.Arc	1.9ab	2.1abc	2.3ab	2.3ab	7
3.Armor	1.6a	1.7a	1.8a	1.9a	2
4.Cimarron	2.2ab	2.3abc	2.4ab	2.4ab	9
5.Endure	1.9ab	2.0abc	2.3ab	2.3ab	7
6.Euver	1.5a	1.7a	1.8a	1.8a	1
7.Maxim	1.8ab	2.0abc	2.2ab	2.2ab	6
8.Pioneer 526	2.5ab	2.6abc	2.6ab	2.6ab	11
9.Raidor	2.1ab	2.3abc	2.4ab	2.4ab	9
10.Saranac AR	2.5b	2.6c	2.7b	2.8b	12
11.Vertus	1.8ab	1.9abc	2.0ab	2.0ab	4
12.WL 320	1.7a	1.8ab	1.9ab	1.9ab	2

[w]MDSR of fifty plants per cv rated on a disease severity rating scale of 1-6.

[x]all cultivars were grown in potting mix B, soil:sand: weblite:vermiculite (2:1:1:1). Two-months-old plants were inoculated.

[y]Ranking of cv according to ascending order of MDSR.

[z]numbers within a column followed by the same letter are are not significantly different according to Student-Newman-Keuls's test for comparison of treatment means ($P \leq .05$).

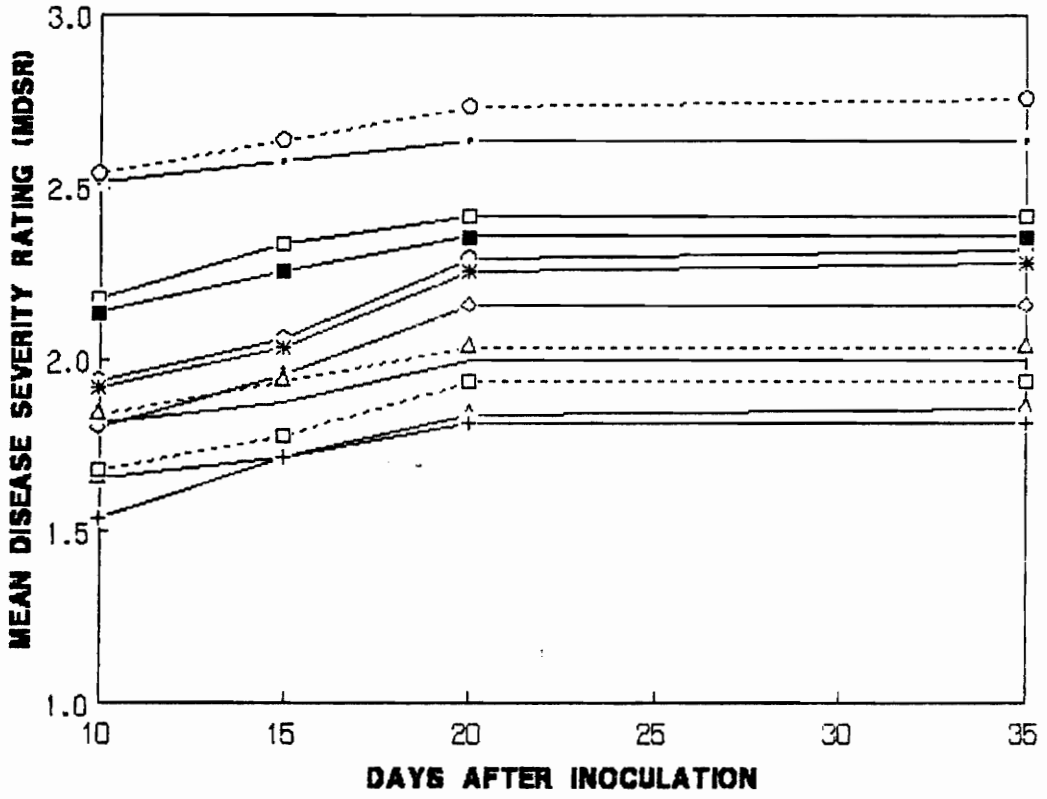


FIG. 15. Progress in disease severity of 12 alfalfa cvs between 10 and 35 days after inoculation with *S. trifoliorum* (isolate LAL 3). Anstar = —, Arc = —O—, Armor = —Δ—, Cimarron = —□—, Endure = —✱—, Euver = —+—, Maxim = —◇—, Pioneer = —•—•—, Raidor = —■—, Saranac = —O—, Vertus = —Δ—, WL 320 = —□—. (Exp. F 1987-potting mix B).

TABLE 22. Mean disease severity rating (MDSR) of twelve cultivars of alfalfa inoculated with five-day-old culture of *S. trifoliorum* (isolate SAL 4) and incubated for 96 hr at 18 C and 100% RH. Exp. G. Date of inoculation-5/5/1987.

Cultivar[x]	[w]MDSR Days After Inoculation 10	15	20	R[y]
1. Anstar	2.5a[z]	2.8a	3.0a	5
2. Arc	3.0a	3.6a	3.7a	12
3. Armor	2.5a	3.0a	3.1a	8
4. Cimarron	3.0a	3.2a	3.3a	10
5. Endure	2.5a	2.8a	3.0a	5
6. Euver	2.6a	2.7a	3.0a	5
7. Maxim	2.5a	2.9a	2.9a	3
8. Pioneer 526	1.6a	1.8a	1.9a	1
9. Raidor	2.5a	2.9a	2.9a	3
10. Saranac AR	2.8a	3.4a	3.5a	11
11. Vertus	2.4a	2.9a	3.2a	9
12. WL 320	1.9a	2.1a	2.1a	2

[w]MDSR of twenty five plants per cv rated on a disease severity rating scale of 1-6.

[x]all cultivars were grown in soil:sand:weblite:vermiculite (2:1:1:1,potting mix B) and and metalaxyl treatment to prevent damping-off. Plants were two-month-old at inoculation and manifested symptoms of phytotoxicity.

[y]Ranking of cvs according to ascending order of MDSR.

[z]numbers within a column followed by the same letter are not significantly different according to Student-Newman-Keul's test for means separation ($P \leq .05$).

Significant differences were observed between the MDSRs at 10 and 20 days but not between 15 and 20 days.

In the regrowth study of experiment D all plants cut back (healthy or those with stem necrosis and / or wilt) put out new stems within a two week period which showed neither stem necrosis nor wilt at the time of observation i.e. 14 days after cutting back. However, the necrotic or wilted stems, themselves, did not regrow. Regeneration ability of the plants in general appeared to be good, which is a favorable characteristic even for infected or colonized plants with respect to stand survival. This condition suggests that the crowns of these plants were not colonized. However, it would have been desirable if an histological examination of the crowns of the inoculated asymptomatic plants as well as of plants having stems with necrotic lesions had been performed to confirm this.

In the re-inoculation study of experiment D (1987) with regrown healthy plants, it was observed that some developed disease symptoms 10 days after re-inoculation. This may be either an indication that some plants escaped infection at the first inoculation or that the crown area of the re-grown stems had rather succulent tissues and hence more susceptible at the time of re-inoculation (i.e. 2 weeks after cutting back) than the original clones after 2-months growth. Thus inoculation of clones obtained from phenotypically non-infected plants may be more useful for further screening.

Analyses of the number of stems per plant per cv in each of the screening experiments revealed that there were significant differences among cvs at 5% level according to SNK test for comparison of means (TABLE 23, 24). However, comparing the order of rankings of cvs according to mean total number of stems with that of MDSR did not show any similarities.

DISCUSSION

Cultivar screening for resistance / susceptibility to *Sclerotinia trifoliorum* can be successfully performed using the mycelial plug inoculation technique under greenhouse conditions as described in the present study. The fact that alfalfa is a heterogeneous crop and no homozygous lines exist is a major reason why cultivars tend to behave slightly differently from experiment to experiment when inoculated with the same isolate of the pathogen under similar conditions. Differences in the mean disease severity ratings (MDSRs) of cultivars were observed even between two experiments carried out within a week's interval on the same potting mix with all other environmental conditions as similar as possible. The range in disease pressure was higher in experiments A & B (1986) in which plants were grown in potting mix A (Soil:peat:vermiculite, 1:1:1) without metalaxyl than in experiments in 1987 in which potting mix B was utilized (soil:sand:weblite:vermiculite, 2:1:1:1) without metalaxyl. The reason for having changed the potting mix in the 1987 experiments was because damping-off of seedlings was a problem with potting mix A and the use of metalaxyl to prevent

TABLE 23. Mean number of stems of twelve cultivars evaluated for disease resistance against *S. trifoliorum* in 1986 in potting mix A.

Cultivar	[x]Mean number of stems/plant			
	Exp.A	R[y]	Exp.B	R
1. Anstar	3.8abcd[z]	6	3.4abc	4
2. Arc	3.6abc	4	2.8a	1
3. Armor	3.7abcd	5	3.4abc	4
4. Cimarr	4.0bcd	10	3.6bc	10
5. Endure	3.9bcd	9	3.2abc	3
6. Euver	3.2ab	3	3.5abc	9
7. Maxim	3.8abcd	6	3.4abc	4
8. Pioneer 526	4.1cd	11	3.4abc	4
9. Raidor	3.1a	1	2.9ab	2
10. Saranac AR	3.1a	1	3.4abc	4
11. Vertus	3.8abcd	6	3.7bc	11
12. WL 320	4.5d	12	3.8c	12

[x]mean number of stems per plant as a means of fifty plants per cv.

[y]Ranking of cv according to ascending order of mean number of stems. C.f. TABLES 14, 15.

[z]numbers within a column followed by the same letter are not significantly different according to Student-Newman-Keul's test for mean separation ($P \leq .05$).

TABLE 24. Mean number of stems of twelve cultivars evaluated for disease resistance against *S. trifoliorum* in 1987 in potting mix B.

Cultivar	[x]Mean number of stems/plant			
	Exp.D	R[y]	Exp. E	R
1. Anstar	3.3a[z]	4	3.6a	3
2. Arc	3.2a	1	3.6a	3
3. Armor	3.6a	9	3.8a	8
4. Cimarron	3.2a	1	3.9a	12
5. Endure	3.6a	9	3.5a	2
6. Euver	3.3a	4	3.7a	7
7. Maxim	3.7a	12	3.6a	3
8. Pioneer 526	3.6a	9	3.8a	8
9. Raidor	3.4a	7	3.8a	8
10. Saranac AR	3.2a	1	3.4a	1
11. Vertus	3.3a	4	3.8a	8
12. WL 320	3.5a	8	3.6a	3

[x]mean number of stems per plant as a means of fifty plants per cv.

[y]Ranking of cv according to ascending order of mean number of stems. C.f. TABLES 17, 18.

[z]numbers within a column followed by the same letter are not significantly different according to Student-Newman-Keuls's test for mean separation ($P \leq .05$).

it produced symptoms of toxicity on the young seedlings. Plants grown in potting mix B alone were uniformly tall and vigorous without neither symptoms of foliar scorch nor damping-off. The higher water holding capacity of potting mix A, due to its peat content, may have been less than favorable for vigor of the plants in the 1986 experiments and been responsible for the higher range in disease severity by enhancing activity of the pathogen. It has been well documented that alfalfa plants under any kind of stress become more susceptible to diseases and this may be true with *Sclerotinia trifoliorum* infection (5). The results of this study confirm the importance having optimum plant vigor free of any stress symptoms at the time of inoculation to prevent confounding MDSR as well as optimum temperature and relative humidity conditions in such evaluation experiments to insure that an inoculation test is neither so severe that promising plants with potentially acceptable resistance are discarded nor a test is not severe enough to detect susceptible plants (5).

All twelve alfalfa cvs were susceptible to all the isolates of the pathogen tested; however, cvs had slightly different responses to them as expected. The differences between the cultivar response to LAL 3 and TAL 4 are not unexpected since these two isolates were determined to have significantly different reactions on cvs Arc and Vertus in the earlier experiments under objective 1. There were differences in virulence of the different isolates. Cultivar response to a

particular isolate appears to be dependent upon the virulence of the isolate, plant vigor and growth conditions at the time of inoculation, and temperature and moisture conditions not only during the incubation period but also afterwards. The high temperature prevalent in the greenhouse and lack of sufficient moisture led to drying out of the colonized tissue of plants in experiment C (1987) which resulted in plants with very poor symptom development. Moisture and temperature are important factors for successful mycelium growth on living tissue (14). It was necessary to top water the inoculated plants in potting mix B very slightly in the greenhouse in later experiments in 1987. This led to very desirable disease symptom development.

Lack of control of temperature within the greenhouse can be a limiting factor since the average remained between 18 C and 29.5 C during the experimental period (Appendix A). It was unfortunate that the inoculations could not have been conducted during the early spring of both years which would have provided more favorable conditions for the development of the disease. However, plants did have optimum temperature conditions during vegetative growth prior to inoculation and optimum temperature and relative humidity conditions after inoculation during the incubation period. It will be, however, desirable to test these cvs using the same inoculation technique and isolates of the pathogen, during cooler temperature conditions of the greenhouse to determine whether there is a better

differentiation of cv response since pathogenicity of the fungus is dependent on cool temperature and high RH. There appears to be a plant genotype-environment-pathogen interaction.

All experiments were conducted at the 96 hr post inoculation incubation period to provide maximum duration of optimum temperature and humidity conditions for infection and also reduce the number of disease escapes. However, the cultivars should also be tested at the 72 hr incubation period, which was shown to allow good differentiation of cvs Arc and Vertus in the preliminary studies under objective 1, to determine whether greater significant differences can be detected among the cultivars.

The method of bottom-watering the plants after removal from the dew chamber was found to be useful in the 1986 experiments utilizing potting mix A but not so in the 1987 experiments with potting mix B. Although bottom-watering of plants may prevent overwhelming disease incidence, may also allow some disease escapes, especially if the water holding capacity of the soil mix is less as with potting mix B. In an experiment conducted comparing the bottom-watering with that of top-watering of cv Vertus plants (potting mix B) inoculated with isolate TAL 4 and incubated for 96 hr, the MDSR produced was significantly greater in the former though only by about 30% (Appendix B, TABLE 26, FIG 16). Greater numbers of plants were infected or dead with top watering. The bottom-watering of inoculated plants, though it may help to control the severity of induced

disease under greenhouse conditions, is a very strenuous task to be undertaken on a routine daily basis, especially when a large number of seedlings is to be evaluated. Thus it is important to investigate this aspect more carefully. A controlled type of misting of plants for the first four to five days after removal from the dew chamber and thereafter top-watering if and when necessary could be a more practical means of conducting large scale evaluation. Should the mycelial plug inoculation technique be found useful for repeated mass screening of alfalfa cvs in the greenhouse, whereby phenotypic selection for resistance can be detected from large populations, it is necessary to make it as easy as possible.

In general, looking at the overall results of the screening experiments, though there was overlapping of MDSRs of the different cvs, with only very slight significant differences, yet a certain amount of repeatability was determined. Cultivar Anstar performed comparatively better in a majority of tests against isolates TAL 4 and LAL 3 but was moderately susceptible to SAL 4. Cultivars Vertus, WL 320, Saranac AR, Endure and Euver were less susceptible to TAL 4 whereas Euver, Armor, and WL 320 were less susceptible to LAL 3. Cultivars Arc, Maxim, and Cimarron appeared moderately susceptible to both isolates. However, cvs Pioneer Brand 526 and Raidor were repeatedly observed to be very susceptible to both isolates under these experimental conditions but fairly resistant to SAL 4. The results with Vertus and Euver are consistent with those

of Raynal (7) who reported that these cvs have high resistance to *S. trifoliorum*. Thus the mycelial plug inoculation technique is found to give reliable results.

It is important to have a reliable and a standard disease severity rating system for such cv screening experiments under greenhouse conditions. The use of 1-6 rating scale based on the percent wilt of individual plants was found to be more useful than the 1-3 scale (7), or the 1-5 scale (3) in detecting significant differences among the cvs though the results obtained with all 3 systems were apparently the same. The simple 1-3 rating scale may appeal especially to a breeder, who needs to screen large populations rapidly. However, the facts that even plants within a cv have different number of stems and that alfalfa has the ability to adjust the number of stems per plant depending on plant density, light intensity, air temperature, soil moisture and fertility all of which affect plant vigor (1) indicates that the degree of infection of plants may tend to change under different growth conditions. This was clearly determined in the present study and was found to be important; thus the 1-6 disease severity rating scale was used as a standard in all experiments and gave reliable results. Harvey (4) developed digrammatic assessment keys for some crown and root diseases of alfalfa, such as bacterial wilt, *Verticilllium* wilt, crown rot by *Fusarium* spp., crown wart caused by *Physoderma alfalfae* and *Stagonospora* root rot. It will be useful to develop such a standard assessment key for

SCSR by *S. trifoliorum* that will enable different personnel to adopt it easily in routine screening studies and the 1-6 disease severity rating scale developed in the present study may serve as a model.

All cultivars tested in all experiments except experiment C of 1987 (which failed) showed progressive increase in disease incidence and severity between the 10 and 20 day observation periods and appeared to show slight differences in the rate of disease progress within this duration. Initial symptoms developed within 15 days and thereafter there were observed changes in the degree of wilt severity per plant. However, the MDSRs remained the same between 20-and 35-day observations which indicates that a maximum of 20-25 days after inoculation should be optimum for significant differences to be detected. Thus rapid results could be obtained three weeks after inoculation with the mycelial plug inoculation technique.

The cultivars with the greatest number of stems did not have the highest disease severity and those with lesser number of stems did not have the least MDSR under these experimental conditions. This observation may not be the same as observed under field conditions, where the microclimate may have a strong influence on the ultimate disease severity and thus a cv with more number of stems could be damaged more due to more humid conditions provided by greater canopy growth. In Cone-tainer® grown plants which were also staked for easy handling and observation of symptoms, the influence of canopy growth on

disease severity may have been negligible.

It would be interesting to compare the anatomical features of greenhouse grown alfalfa plants with those of field grown plants of the same age to determine whether there are any which may play a role in the disease resistance mechanism. It should be also useful to investigate any differences in the physiology of the disease resistance mechanism of these cvs. Scott (10) reported differences in the ability of different isolates of *S. trifoliorum* to cause disease on different legume hosts. He pointed out that the production of polygalacturonase and ∝-L-arabinofuranosidase was involved in addition to pectin esterase and pectin lyase in the disease development caused by *S. trifoliorum* on alfalfa and clovers. Thus this host-pathogen system appears to be extremely complex. Such studies based on these particular isolates will enable one to decide which isolates can be used in screening material for resistance to SCSR.

The mycelial plug inoculation technique could serve as a primary 'sieve' in obtaining phenotypically resistant germplasm (apparently healthy plants) which could be increased in population and re-inoculated. The survivors from the re-inoculation test could be grown further to produce seeds via mass selection which then should be tested in the greenhouse and compared with the original parent seed stock to determine whether any progress toward developing resistant germplasm has been made, before field testing. The technique appears promising, and may play a role in a breeding program for SCSR resistant alfalfa cultivars.

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CHAPTER 4

SUMMARY

Sclerotinia crown and stem rot (SCSR) of alfalfa (*Medicago sativa* L.) caused by *Sclerotinia trifoliorum* Eriksson has been determined to be the cause of severe stand failures, especially in fall-seeded, no-tillage plantings, in Virginia in recent years. At present no economical and highly effective control practice is available for this disease. Planting of disease resistant plants has been one of the more economic and practical means of disease control in other agricultural crops. Since alfalfa, unlike most agronomic crops, is genetically very heterogeneous, breeding for disease resistance is inherently more difficult. No alfalfa cultivar (cv) of agronomic importance is currently known to be highly resistant or immune to *S. trifoliorum*. All cvs under favorable disease conditions succumb to SCSR.

Evaluation of alfalfa cvs under natural field conditions, although favorable is unreliable due to the erratic occurrence of the disease. However, several artificial inoculation techniques have been tried to evaluate alfalfa cvs for resistance to *S. trifoliorum* . In one such technique, a mycelial plug inoculation procedure, a plug removed from the margin of the actively growing pathogen on a solid medium, is placed at the crown area of two-month-old alfalfa plants. Inoculated plants are incubated under favorable temperature and relative humidity conditions for a pre-determined period of time.

In the present study, under objective one, it was determined, by mycelial plug inoculation technique that isolates differed in their virulence towards two alfalfa cvs Arc and Vertus under similar experimental conditions. Isolates TAL 4 and TAL 2 were significantly more virulent than CAL 1, CAL 3 and LAL 3 on both cvs and a 96 hr incubation in the dew chamber produced significantly higher disease severity than a 48 hr period. In general, cv Vertus was less damaged than Arc by isolates LAL 3, and CAL 3, while TAL 4 was equally severe on both. Inoculation of the two cvs with the isolates tested gave typical SCSR symptom expression, a characteristic favorable to a good evaluation test. Post inoculation incubation temperature and relative humidity (RH) are essential in disease establish-ment. In the present study, incubation of inoculated plants in a dew chamber at 18 C and 100% RH for either 24, 48, 72 or 96 hr, provided adequate conditions to promote infection and colonization of inoculated plants. The 96 hr incubation produced significantly greater disease severity on both cvs. Inoculation with isolates LAL 3 and CAL 3 distinguished between the two cvs better than TAL 4 for this incubation time. However, at the 72 hr incubation period, all three isolates differentiated between cvs. The fact that the 96 hr incubation period and isolate TAL 4 produced significantly greater disease severity without killing all plants, led to the selection of this incubation time and isolate for the cv evaluation tests under objective 2. Cultivar evaluation was also conducted with the

less virulent isolate LAL 3.

A study of age of alfalfa plant in relation to inoculation time was conducted. At 20-days after inoculation, 4-, 6-, and 7-week-old seedlings of cvs Arc and Vertus were severely damaged or killed. Five-, eight- and nine week-old plants were less susceptible but did produce adequate symptom expression. However, this age study should be repeated with plants with uniform vegetative growth since test plants suffered from metalaxyl toxicity. Since inoculation of plants older than two months failed to produce sufficient symptom expression, mature plants are more resistant to the pathogen and thus would not be useful for cv evaluation studies.

Evaluation of cvs for disease resistance, under greenhouse conditions, with artificial inoculation techniques may produce disease symptoms not observed under field conditions. Thus it is important to observe and interprete disease reactions carefully. In the present study, the symptoms produced upon inoculation of plants with plugs of isolates of *S. trifoliorum*, closely resembled the SCSR symptoms obtained under natural field infection. These symptoms were observed within a period of twenty days after inoculation with no further changes thereafter. This is a favorable feature in the development of a successful inoculation technique since it will permit evaluation to be completed in a relatively short period of time. However, individual inoculated plants have to be examined with care since alfalfa plants within a cv tend to differ in the number of stems

per plant and genotype. Care should be taken not to overlook disease resistant germplasm. Plant age, architecture, environmental conditions such as temperature, relative humidity and light during and after incubation play a role in the rate and degree of disease symptom development. Though these conditions were kept a constant as possible, there were slight variations among experiments due to unavoidable circumstances.

Under objective two, in which the same twelve cultivars of alfalfa were tested for disease resistance / susceptibility to *S. trifoliorum* isolates, all cvs were determined to be susceptible to the isolates tested, but they, however, showed differences in susceptibility. Although no cv exhibited an outstanding degree of resistance (eg. immunity) in all experiments, Anstar followed by WL 320, Vertus, Saranac AR were less susceptible to the virulent isolate TAL 4 in a majority of the tests. With the less virulent isolate LAL 3, Armor and Euver were less susceptible and Anstar also showed good tolerance while Saranac AR was observed to be highly susceptible. Cultivars Pioneer Brand 526 and Raidor were observed to be highly susceptible to TAL 4 and LAL 3 but were less susceptible to SAL 4. Cultivars Euver, WL 320, Endure, Arc and Armor showed fairly good tolerance in some of the tests.

Proper selection of a suitable isolate for cv evaluation studies may be an extremely important cosideration since it has been determined that different isolates differed in their virulence towards different alfalfa cvs as well as other legume

hosts. Cultivar responses may differ depending on the nature of pathogenicity of the isolate and the host genome. Under field conditions, ascospores are the source of primary inoculum and leaves of young seedlings are the site of infection, as a result, there are bound to be differences between the disease responses of these cvs under field and greenhouse conditions. It is thus essential to test these cvs against inoculations using ascospores of either any one of the virulent isolates or a mixture of isolates, of *S. trifoliorum*, as inoculum. The production of large numbers of ascospores for such a purpose is very essential and should be a priority in future research.

The variation in cv responses to *S. trifoliorum* (isolate TAL 4) among the four experiments conducted under objective 2 may be due to slight differences in plant vigor, soil moisture conditions, environmental conditions (post incubation) and especially due to the highly heterogeneous nature of the crop.

Alfalfa is not like most crops, such as corn or wheat, in which homozygous disease resistant germplasm may be selected following such a technique. This may be a disadvantage of this inoculation technique in which the site of inoculation (crown area) and source of primary inoculum (mycelium as opposed to ascospore) are different from those of natural field infection of alfalfa by SCSR caused by *S. trifoliorum*.

However, by the use of the mycelial plug inoculation technique in which individual plants are inoculated, potential disease resistant germplasm in the form of asymptomatic plants

(apparently healthy) can be selected for propagation, seed production and re-evaluation in a phenotypic selection progress for obtaining SCSR resistant alfalfa cvs. The differences in disease severity among plants of a cv indicates the presence of resistant genes. This step of selecting disease free or symptomless plants after exposure to a pathogenic isolate of *S. trifoliorum* will act as a 'sieve' in selecting plants of cvs for further testing under greenhouse and field conditions which is of prime importance.

The success of this technique depends on the virulence of pathogen isolates, the uniformity of test plants, the temperature and RH held during and after incubation, the regulation of the moisture regime of inoculated plants and the adoption of a standard disease severity rating scale. The 1-6 disease severity rating system used in these experiments was found to be more reliable than the 1-3 or 1-5 scales in detecting significant differences in the disease responses among cvs and can serve as a model for future work. The absence of a standard rating system can lead to much variation in the results obtained in such an inoculation technique. This mycelial plug inoculation technique to distinguish differences in disease responses of different alfalfa cultivars to different isolates of *S. trifoliorum* is reliable and repeatable. It can be used for evaluation of cvs on a large scale under greenhouse conditions. This technique will allow the breeder to test large number of plants from a wide range of cvs within a short period

of time and select disease resistant germplasm for masss selection and further evaluations. It is believed that, based upon the data obtained in this study, progress could be made towards the development of SCSR resistant alfalfa cultivars.

Appendix A TABLE 25.

Hygrothermograph Temperature and Relative humidity recordings in the greenhouse 1987 experimental period.

Date	Temperature C			Relative Humidity %		
	Max.	Min.	Av.	Min.	Max.	Av.
March						
6	30	19.5	24.75	25	47	36
7	25	20	22.5	28	48	38
8	27	22	24.5	24	60	42
9	27	18	22.5	50	80	65
10	20	15	17.5	52	35	43.5
11	29	18	23.5	22	58	40
12	29	23	26	34	58	46
13	28	16	22	22	45	33.5
14	24	12	18	25	40	32.5
15	25	22	23.5	30	50	40
16	22	21	21.5	36	87	61.5
17	22	22	22	44	66	55
18	23	19	21	32	50	41
19	29	23	26	30	45	37.5
20	30	20	25	30	100	65
21	26	20	23	35	10	22.5
22	23	20	21.5	28	34	31
23	27	21	24	15	29	22
24	27	20	23.5	26	35	30.5
25	26	23	24.5	26	32	29
26	24	23	23.5	31	62	46.5
27	32	23	27.5	25	45	35
28	-	-	-	-	-	-
29	-	-	-	-	-	-
30	-	-	-	-	-	-
31	-	-	-	-	-	-
April						
1	-	-	-	50	80	65
2	-	-	-	32	55	43.5
3	-	-	-	34	73	53.5
4	-	-	-	47	80	63.5
5	-	-	-	43	100	71.5
6	-	-	-	35	100	67.5
7	-	-	-	33	77	55
8	39	23	26	32	60	46
9	40	20	30	50	75	62.5
10	35	21	28	38	50	44
11	35	21	28	12	47	29.5
12	35	21	28	40	49	44.5
13	32	21	26.5	25	46	35.5
14	36	17	26.5	38	74	56
15	27	20	23.5	26	76	51

Date	Temperature C			Relative Humidity %		
	Max.	Min.	Av.	Min.	Max.	Av.
16	29	22	25.5	62	86	74
17	26	22	24	65	90	77.5
18	33	22	27.5	36	63	49.5
19	34	21	27.5	33	65	49
20	35	21	28	26	65	45.5
21	37	21	29	27	64	45.5
22	37	21	29	30	79	54.5
23	22	21	21.5	67	100	83.5
24	21	20	20.5	65	95	80
25	21	21	21	70	95	82.5
26	31	21	26	17	55	36
27	28	20	24	25	75	50
28	30	19	24.5	20	60	40
29	33	18	25.5	20	55	37.5
30	33	17	25	09	50	29.5
May						
1	32	17	24.5	22	65	43.5
2	31	18	24.5	30	80	55
3	33	17	25	74	95	84.5
4	31	12	21.5	24	55	39.5
5	36	13	24.5	14	70	42
6	35	13	24	20	65	42.5
7	37	15	26	12	60	36
8	35	15	25	17	70	43.5
9	36	16	26	17	74	45.5
10	37	17	27	28	80	54
11	40	17	28.5	30	83	56.5
12	31	19	25	52	84	68
13	27	18	22.5	74	100	87
14	32	18	25	55	98	76.5
15	31	17	24	55	100	77.5
16	33	17	25	32	80	56
17	35	18	26.5	33	100	66.5
18	33	18	25.5	50	100	75
19	32	18	25	55	93	74
20	27	17	22	70	100	85
21	28	19	23.5	60	100	80
22	27	18	22.5	60	95	77.5
23	31	18	24.5	45	92	68.5
24	27	18	22.5	57	92	74.5
25	28	18	23	48	90	69
26	29	18	23.5	49	92	70.5
27	29	18	23.5	52	90	71
28	30	21	25.5	62	90	76
29	29	17	23	48	90	69
30	28	17.5	22.75	53	91	72
31	27	18	22.5	55	90	72.5

Date	Temperature C			Relative Humidity %		
	Max.	Min.	Av.	Min.	Max.	Av.
June						
1	26	17	21.5	60	91	75.5
2	29	17	23	38	86	62
3	32	19	25.5	35	87	61
4	26	16	21	60	95	77.5
5	26	16	21	40	86	63
6	29	17	23	42	90	66
7	30	17	23.5	42	92	67
8	28.5	18.5	23.5	44	93	68.5
9	28	18	23	55	95	75
10	27	19	23	46	85	65.5
11	27	18.5	22.75	56	100	78
12	25	17.5	21.25	71	95	83
13	26	15.5	20.75	64	91	77.5
14	26	15	20.5	50	90	70
15	26	16.5	21.25	55	90	72.5
16	24	14	19	64	90	77
17	24.5	14	19.25	55	90	72.5
18	23	16	19.5	70	90	80
19	28.5	18	23.25	60	100	80
20	27	18.5	22.75	68	95	81.5
21	28	18	23	63	95	79
22	28	18.5	23.25	63	95	79
23	29	18	23.5	59	94	76.5
24	31	20	25.5	45	95	70
25	25.5	20	22.75	67	98	82.5
26	29	16	22.5	58	100	79
27	31.5	15	23.25	28	78	53
28	32	15.5	23.75	23	85	54
29	35	18	26.5	32	95	63.5
30	37	18.5	27.7	26	80	53
July						
1	32.5	20	26.25	43	93	68
2	29	19	24	60	95	77.5
3	31	21	26	60	100	80
4	31	20.5	25.75	57	96	76.5
5	33.5	20.5	27	46	95	70.5
6	35	20.5	27.75	50	95	72.5
7	39	20	29.5	47	95	71
8	35	21	28	45	100	72.5
9	34.5	22	28.25	50	100	75
10	32	20.5	26.25	52	95	73.5
11	33.5	20	26.75	47	94	70.5
12	30	18	24	49	93	71
13	31	19	25	47	92	69.5
14	28	14.5	21.25	43	90	66.5
15	23	14.5	18.75	50	90.5	70.25

Date	Temperature C			Relative Humidity %		
	Max.	Min.	Av.	Min.	Max.	Av.
16	26.5	14	20.25	34	88	61
17	31	18	24.5	48	96	72
18	33	18	25.5	38	95	66.5
19	33.5	18.5	26	41	95	68
20	35	20	27.5	40	95	67.5
21	37	20.5	28.75	42	97	69.5
22	36	22	29	37	95	66
23	35.5	21	28.25	45	98	71.5
24	36.5	20	28.25	39	95	67
25	37	21	29	31	94	62.5
26	37.5	22	29.75	30	93.5	61.75
27	34	21.5	27.75	44	95	69.5
28	33	20	26.5	38	93.5	65.75
29	33	20	26.5	38	96	67
30	34.5	20.5	27.5	40	96	68
31	33.5	20	26.75	42	96	69

Appendix B. Effect of different types of watering on the disease severity on an alfalfa cultivar inoculated with a 5-day-old culture of *S. trifoliorum* and incubated for 96 hr at 18C and 100% RH.

Introduction

Sclerotinia crown and stem rot (SCSR) caused by *Sclerotinia trifoliorum* is severe on alfalfa and clovers at temperatures between 15 C and 18 C and at a relative humidity of about 90% or greater. Thus it is important to provide ideal temperature and humidity conditions for disease development when artificial inoculation techniques are used to evaluate for disease resistance under greenhouse conditions. Even though these ideal conditions can be given during the incubation period, it is essential to control these factors after removal from the dew chamber. It is necessary not to have overwhelming disease incidence and severity of symptoms when screening for resistance of alfalfa because all cultivars succumb to the disease and potential resistant germplasm could be lost. On the other hand, if the conditions are not severe enough there will be a greater number of disease escapes. With these criteria in view, two types of watering methods, top-vs bottom-watering, were tested on the disease severity on alfalfa cv Vertus, in order to have controlled moisture regimes after incubation of inoculated plants.

MATERIALS AND METHODS

Alfalfa cv Vertus plants were grown in Cone-tainers®

containing potting mix B, soil:sand:weblite:vermiculite (2:1:1:1), lime, and Rhizobium inoculant. Five seeds per Conetainer® were seeded but later thinned to one seedling at 12 days. P and K Fertilizer was applied as 1ml/Conetainer® of a solution of 13.1g KCL and 19.6g Na_2PO_4 per liter. The two treatments were (1) top-watering and (2) bottom-watering. Two sets of 50 plants (two-month-old) per treatment were inoculated with 6mm plugs from the margin of 5-day-old culture of a virulent isolate *S. trifoliorum* (TAL 4) as described in Chapter 2. The date of inoculation was June 5, 1987. The inoculated plants and the non-inoculated controls (10 per bottom-watering and 2 per top-watering) were randomly arranged in trays which were then randomly placed in the dew chamber and incubated for 96 hr at 18C and 100% RH. All non-inoculated control plants were grown in tared Cone-tainers® (including potting mix). All planting media were brought to saturation prior to inoculation. Upon removal from the dew chamber the plants were arranged into the respective treatments (trt.) and maintained on the same greenhouse bench which received 9 hr (3:00pm-12:00 am) of supplemental light from a sodium vapor (HPSV) lamp (1000 watt). Post incubation the plants were watered daily. The control plants of trt. 2 which received bottom-watering were tared before and after watering daily. The tray of plants were placed in a Cone-tainer® with a standard volume of water (26 liters) for five minutes daily (about 1/3-1/2 the Cone-tainer® immersed). The amount of water taken up by each control plant was determined. The 2 control

plants of trt. 1 (top-watering) were also tared before and after watering. In this case, the average amount of water gained (ranged from 3ml-10ml) by the 10 control plants of trt. 2 was applied to trt. 1 plants by means of a pipette. The experiment was conducted for 20 days after inoculation at which time the final disease rating was taken. Disease symptom ratings for SCSR were recorded on a scale of 1-6 (as previously described in the other expts.) at 10, 15 and 20 days after inoculation. The data were analysed by suitable ANOVA procedure and the Student-Newman-Keul's (SNK) mean separation test.

RESULTS

The plants of both treatments were uniformly vigorous at the time of inoculation (no foliar disorder or stem wilt). On removal from the dew chamber (D.C.) mycelium growth was visible on the inoculated plants of both treatments but not on the control plants. There was adequate dew formation on both treatments in the dew chamber. During the entire period of the experiment, plants of trt.1 (top-watering) appeared to be more diseased than those of trt. 2. The soil surface of trt. 1 plants remained moist whereas those of trt. 2 remained consistently dry and the crown area did not become moist on any day after watering. The rate of development of disease symptoms in trt. 1 was greater. The mean disease severity rating of trt. 1 was significantly higher than that of trt. 2 (bottom-watering) on all three observation dates, though only by a small magnitude (TABLE 26, FIG. 16).

TABLE 26. Effect of two types of watering (post incubation) on the disease severity on alfalfa cv Vertus inoculated with *Sclerotinia trifoliorum* (isolate TAL 4) and incubated in the dew chamber at 18 C and 100% RH for 96 hr.

Days After inoculation	[w]Mean Disease Severity Rating (MDSR)	
	1	2
	[x]Top-Watering	[y]Bottom-Watering
10	2.6b[z]	1.8a
15	2.7b	1.9a
20	2.7b	1.9a

[w] MDSR of 50 inoculated plants per treatment. Plants were grown in potting mix B.

[x] Top watering: individual plants received average volume of water gained by the 10 control plants of treatment 2.

[y] Bottom-watering: tray of plants were kept in a large pan of standard volume of water for 5 minutes daily.

[z] numbers within rows followed by the same letter are not significantly different according to Student-Newman-Keul's test for mean separation ($P \leq .05$).

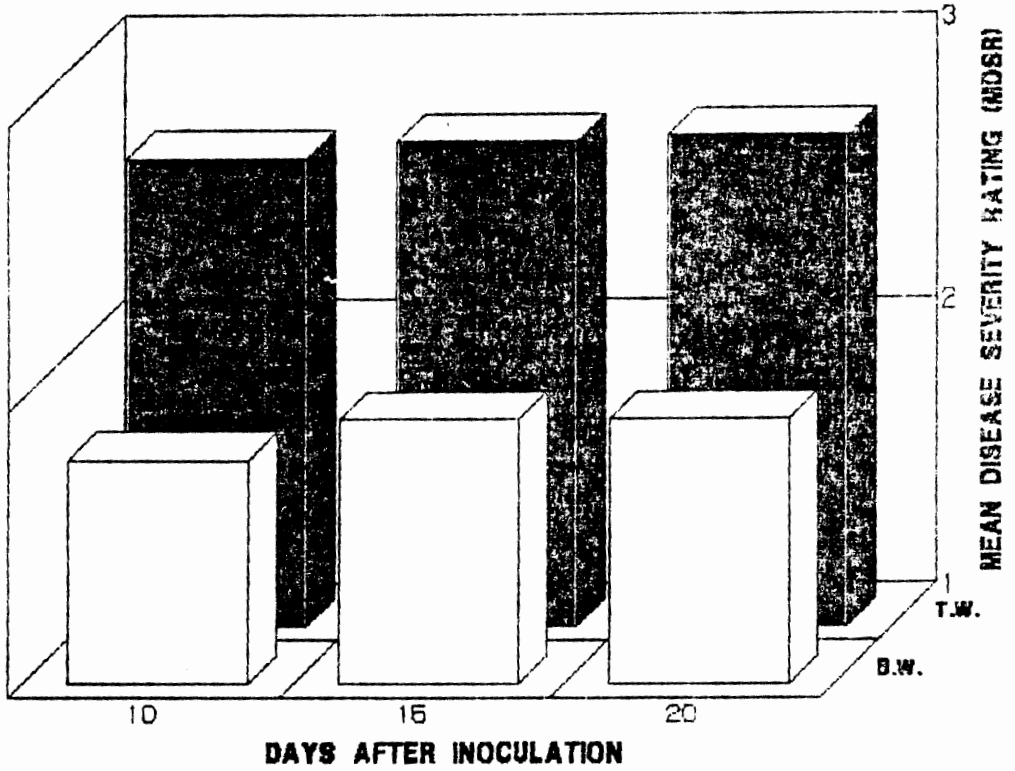

FIG. 16. The effects of two different types of watering techniques, bottom-watering (B. W) vs. top-watering (T.W.) on the disease severity of alfalfa cv Vertus inoculated with *S. trifoliorum* (isolate TAL 4) Potting mix B.

CONCLUSION

Top-watering of plants provided more favorable conditions for the development of SCSR symptoms on Vertus inoculated by the mycelial plug technique and under the specific experimental conditions stated above. Also top-watering of plants without drenching the foliage will provide suitable moisture conditions for the development and establishment of *S. trifoliorum* on alfalfa in inoculation studies with the objective of screening many cultivars. The bottom-watering technique may prove adequate when plants are grown in soil:peat:vermiculite mix (potting mix A-1986) but tends to be tedious and impracticable for routine greenhouse work of this nature. In this study, the top-watering treatment produced a greater number of infected and dead plants than bottom-watering did. However, though the rate of disease development and disease severity were greater with top-watering, the disease was not so overwhelming as to kill all the plants. Therefore light top watering of plants inoculated with the mycelial plug technique without drenching is adequate to obtain good disease pressure when soil:sand:weblite:vermiculite mix is utilized. However, a more practical method of light top-watering of plants in order to keep the crown area moist should be investigated since under natural field conditions alfalfa crop is not irrigated in Virginia.

VITA

The author was born and raised in Vaddukoddai, Jaffna, Sri Lanka (Ceylon). She had her entire school education at Jaffna College, Vaddoukoddai. She obtained the General Certificate of Education Examination conducted by the Department of Education, SriLanka, with advanced level passes in Botany, Chemistry, Zoology and Physics. She received her Bachelor of Science Degree in January 1974 Botany with Second Class Honours, and Chemistry as the the subsidiary subject, from the University of Sri Lanka, Peradeniya. She worked as an assistant lecturer in Botany for three years in the universities of Sri Lanka, Peradeniya and Jaffna. Since August of 1977, She has been attached to the Department of Agriculture of Sri Lanka, first as an Experimental Officer and later as a Research Officer in in Plant Pathology, in the Northern Region. Her Master of Science degree was received in January 1988 from Virginia Polytechnic Institute and State University, Department of Plant Pathology, Physiology and Weed Science, with a major in Plant Pathology. The author gained work experience in the Plant Disease Clinic during the summer of 1987. She is a member of the Virginia Tech Chapter of Gamma Sigma Delta Honour Society.