Proof of pathogenicity of Fusarium slani (Mart.) Appel & Wr. to sainfoin (Onobrychis viciifolia Scop.) and development of procedures to select for resistance
by Dick Lindsey Auld

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Crop and Soil Science
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Abstract:
Root and crown rot limits the production of sainfoin. This disease reduces longevity and forage yield. The objectives of this study were to: 1) determine the causal organism(s) of root and crown rot; 2) further characterize the host-parasite relationships; and 3) develop and initiate screening procedures to detect potential sources of disease resistance.

The results of this study indicate that the root and crown rot of sainfoin usually observed in Montana is caused by Fusarium solani. This pathogen is not seed transmitted but probably occurs in most agricultural soils as a pathogen of other legumes.

Sainfoin can be successfully screened for disease resistance by soaking injured roots in concentrated suspensions of microconidia of F. solani. Initial screening to locate potential sources of disease resistance has shown differential disease resistance in both the world collection of Onobrychis and current breeding material. Selection within these populations should increase disease resistance.

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DICK LINDSEY AULD

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Approved:

Raymond L. Patterson
Chairman, Examining Committee

Kurt C. Feltner
Head, Major Department

Henry L. Parsons
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

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ABSTRACT

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INTRODUCTION

Sainfoin (Onobrychis viciifolia Scop.) is a non-bloating perennial legume which has been used for hay production and pasture in Europe for several centuries. Cultivation of sainfoin began in Montana in 1964 with the release of 'Eski'. Sainfoin rapidly gained popularity because of its excellent forage quality, winter hardiness, drought tolerance, palatability, and resistance to the alfalfa weevil (Hypera postica Gyllenhal). It offered an excellent alternative to alfalfa (Medicago sativa L.).

In recent years sainfoin has been attacked by a root and crown rot which limits persistance and reduces forage production. This disease is found statewide and is thought to be caused by Fusarium spp. Sainfoin's popularity has declined because of root and crown rot. Disease resistant varieties must be developed if sainfoin is to remain an economic forage crop.

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LITERATURE REVIEW

Description of Onobrychis viciifolia Scop.

Sainfoin is a deep rooted, perennial, forage legume. It has a branched tap root system which may extend to a depth of several meters and be 5 cm in diameter (60). Nodulation occurs on the fine lateral roots and the young tap roots (3,54,69).

Multiple erect stems, 90-150 cm tall, arise from a branched, prostrate crown. These stems are usually hollow and bear pinnately, compound leaves with 13-15 leaflets per leaf (18,60).

Sainfoin's influorescence is an erect raceme with from 5-80 florets. Each floret gives rise to single seeded pod (6,42). The pods are brown, indehiscent, lenticular, and reticular on the surface (60). The seed are brown to black in color and are approximately 2.5 mm long, 2.0-3.5 mm wide, and 1.5-2.0 mm thick (6). The seed pod comprises approximately 30% of the weight of the pod-intact seed (6,16).

History and Distributions of Onobrychis viciifolia Scop.

Sainfoin has been utilized as a forage crop in Russia for over 1,000 years and in Europe for over 400 years (60,68). Early immigrants brought plants of this species to the North American Continent with introduction into Montana dating back to before 1900 (18).

Sainfoin was evaluated as a potential forage crop in the early part of this century (60), but failed to develop into a major crop

because of disease problems; failure to recognize its forage quality, palatability, and non-bloating characteristics; and due to testing in areas not well adapted to this crop. In recent years, the increasing losses of alfalfa to the alfalfa weevil, and the need for a higher yielding, non-bloating forage legume, has increased the interest in sainfoin.

In 1964 the variety Eski was released by the Montana Agricultural Experiment Station. This variety was selected from plant introductions from Turkey (19). Other varieties such as 'Onar' and 'Melrose' are selections from plant introductions from Russia (28). Currently, plant breeders working in sainfoin are continuing to evaluate both new plant introductions and the earlier introductions which have become adapted to this country.

Agronomic Characteristics of *Onobrychis viciifolia* Scop.

Sainfoin is drought tolerant, winter hardy, and adapted to coarse calcarous soils, which are not well suited for other legumes (31,37,60). It has been utilized for hay production, seed production, and pasture under both dryland and irrigated conditions. However, many researchers report there is a lack of effective nodulation and that stand persistence is poor under certain conditions (3,48,54,66, 69).

Sainfoin has high quality forage and has never been reported to cause bloat (6,16,18,28,60). Feeding trials conducted in Canada, Montana, Idaho, and Nevada have shown sainfoin hay to equal alfalfa hay in nutritive value and to have even greater palatability (4,7,27, 28,36,55). Sainfoin seed also has the potential of becoming a protein supplement in animal diets (16).

Hay yields of sainfoin have been variable. When grown under proper conditions, yields of sainfoin are competitive with the best adapted alfalfa varieties (4,7,27,28,36,55). Best yields are obtained on well-drained, basic soils with a good moisture-holding capacity (4,18,28,60). Sainfoin is sensitive to frequent cuttings. Most existing varieties produce maximum forage in the first cutting (4,18,55). Many stands begin to fail in the third year when irrigated, and in the fourth or fifth year under dryland conditions (4,28,31,62). This stand depletion is thought to be the result of a disease which severely damages the crowns and roots of mature plants (48,66).

Seed yields often approach 1,500 kilograms of clean seed per hectare (27,55). In colder climates, seed is usually not harvested until the second year of establishment. Maximum seed yields are obtained if the first cutting is allowed to set seed (6,28,55).

Sainfoin's role as a pasture legume is enhanced by its lack of bloat, good feed value, and broad range of adaptation (18,28,43). It can either be pastured alone or in mixtures with grasses such as

Russian wild rye-grass or crested wheatgrass. However, it does not seem to compete well with aggressive, rhizomatous grasses such as brome grass or pubescent wheatgrass (28,43). The use of sainfoin in the hay-stockpile management regime distributes forage production and allows an increase in total carrying capacity (12).

Diseases of Onobrychis viciifolia Scop.

Foliar diseases. Most work on foliar diseases has been done in Europe. Mathre (48) in a review, found reports of several foliar diseases of sainfoin. A leaf spot caused by the fungus Ramularia onobrychidis Allescher, forms dark brown spots on the upper surface of leaves. This disease is most severe under moist conditions and occurs in large areas of Europe (33,48).

Another leaf spot, which occurs primarily in England, is caused by the fungus Septoria orobina Saac. It forms fawn-colored spots on both the leaves and young pods. This disease may be disseminated by contaminated pods (34,48).

Ring spot, which occurs in both England and Montana, is caused by the fungus Pleospora herbarum (Pers.) Rabenh. The symptoms are similar to those of Septoria leaf spot. Conidia form under moist conditions to promote rapid spread. The disease does not appear to cause economic damage in Montana (32,48).

The fungus Aschochyta onobrychidis forms spots on both the leaves and stems. It is found in Europe and Montana, where it occurs primarily as blackened stem lesions. This fungus appears to be seed borne and persists on crop residue. It is not considered to be causing economic damage (32,48).

Other diseases attacking the foliage of sainfoin are: a rust caused by Uromyces onobrychidis; chocolate spot caused by Botrytis conerae; and powdery mildew caused by Erysiphe polygoni D.C. (32,48). None of these foliar diseases are thought to be of economic importance in Montana (48).

Soil-borne diseases. Diseases, that attack the crowns and roots of sainfoin, are the primary cause of stand reduction. Verticillium wilt of sainfoin (Verticillium albo-atrum Reinke and Berth) has been observed in Europe and causes wilting and vascular discoloration (35). It is most severe during the warmer months when the plant is often under moisture stress. This pathogen has not been isolated from diseased sainfoin plants in Montana (48,66).

The fungus, Sclerotinia trifoliorum Erikss, causes root, crown, and stem rot in Montana and Europe (32,48). Infected plants wither and turn brown during the middle of the growing season and sclerotia can be found in the pith tissue. Under high moisture conditions, white or tan mycelial mats appear near the soil line. The damage in infested

fields is usually light in Montana, with only 1% of the plants showing symptoms (48).

Rhizotonia solani Kuehn. has been isolated from secondary roots and darkened lesions on tap roots. R. solani has never been isolated from the internal portion of the crown but could possibly act as a source of entry for other pathogens (66).

Sainfoin is extremely susceptible to the northern root-knot nematode, Meloidogyne hapla Chitwood. Damage was more severe at higher temperatures. Unlike alfalfa, both mature and young roots of sainfoin are susceptible (24).

The most serious disease of sainfoin in Montana is root and crown rot though to be caused by Fusarium spp. (48,66,67).

Seed and seedling diseases. The seed and seed pod of sainfoin has been shown to carry Alternaria, Fusarium, Mucor, and numerous bacteria (66,74). Many of the bacteria were antagonistic to nodulation (74). Contaminated seed have been shown to spread Fusarium pathogens in beans (Phaseolus vulgaris L.) and mimosa (Albizia julibrissin) (22,58).

Sainfoin seedlings are susceptible to Alternaria spp., Rhizoctonia solani and Pythium spp. (66). Symptoms often appear as classical 'damping off'.

Development of Root and Crown Rot in Onobrychis viciifolia Scop.

Occurrence of the disease. Sears (66,67) in an extensive study of Montana found the root and crown rot of sainfoin in nine of ten sampled locations. While both F. solani (Mart.) Appel & Wr. and F. oxysporum Schlecht. were isolated, F. solani was present in over 90% of the diseased plants. This disease also causes severe losses in New Mexico and may occur in other areas where sainfoin is grown (52).

When Eski was grown under normal field conditions, F. solani was isolated from 60% of the seedlings by the end of the first growing season. The rapid entry of this fungus was thought to be enhanced by the seed pod wounding the young tap root as it began to thicken and elongate (66). The seed pod reduces emergence in the field and germination in the laboratory (5). Removal of the pod prior to planting may be beneficial (5,66).

Symptoms begin to appear during the second year. The crown appears to be unable to support the increasing number of stems and splits. This tearing may be a source of entry to pathogens. F. solani has been consistently isolated from this area. The disease appears to develop first in the stem, just above the crown where it forms a darkened streak which extends into the vascular tissue of the crown. As the disease progresses, the crown tissue is destroyed resulting in the eventual death of the plant (66). Fumigation of field plots increased vigor and regrowth, and reduced the frequency of root and crown rot in

sainfoin (66). The crown splitting does not result in destruction of crown tissue in the absence of pathogens.

Occurrence and severity of root and crown rot of sainfoin could be aggravated by root damage caused by nematodes or insects. In alfalfa, the infection of F. oxysporum was increased from 15 to 90% by the addition of northern root-knot nematodes (Meloidogyne hapla Chitwood)(51). In red clover (Trifolium pratense L.) insect root injury increased the incident of crown rot (23). Non-parasitic nematodes such as Diplogaster lheritieri Maupas may spread plant pathogens. These nematodes injest spores of F. oxysporum and Verticillium spp. without reducing their viability (37).

The suspected pathogen. F. solani is a common pathogen of most agronomic crops (2,17,47). This fungi exists in the soil and in crop residue as chlamydospores. Chlamydospores are resting structures produced by hyphae and conidia through the condensation of their contents and the formation of a thick wall (56,72). Root exudates of susceptible plants stimulate the chlamydospores to germinate and produce mycelium which seek the surface of the root (11). The fungus forms a small surface thallus and penetrates the plant through the middle lamella at a junction of epidermal cells (9,11). It also invades roots through mechanical and natural wounds. Once established, hyphae invade the living cortex intercellularly until they are stopped by the endodermis (11).

When the hyphae are exposed to daylight they produce macroconidia and microconidia. Macroconidia are large sickle-shaped condia with three to seven cells and are usually formed on sporodichia. Microconidia are produced on the aerial mycelium and are usually composed of only one or two cells (72). The principle function of the spores is dissemination, but they may also be directly infective (57). When macroconidia are added to the soil they form a short hyphae which then forms chlamydospores (11,56).

Isolates of F. solani may produce a hormonal growth response in plants that are grown in infested soil (11,25,56,66) The asexual forms are capable of genetic recombination through the processes of heterokaryosis and parasexuality (50,72).

The genus Fusarium is divided into nine taxonomic species in a system developed by Snyder and Hansen (72). This system uses the shape and morphology of macroconidia as its principle index. Other characteristics such as colony morphology and pigmentation are undependable and subject to rapid mutation (72). F. solani has been divided into nine formae species on the basis of selective pathogenicities to their hosts (50). This selectivity is subject to rapid mutation and may be simply inherited (73). Some investigators feel the division into formae species is highly artificial and question its validity (61).

F. solani has been a severe pathogen on beans (2,49,56), peas (Pisium sativum L.)(47,49), and many other legumes (20,49,56). This fungus produces a sexual stage (Hypomyces) that is composed of individuals which are self-sterile, interfertile, and usually hermaphroditic. Ascospores are found in perithecia which develop in nature only on mulberry leaves under very humid conditions. Investigators recently induced the sexual stage in vitro (49,50).

Crown Rot in Other Forage Legumes

Red clover has historically been limited by a lack of stand persistence due largely to root and crown diseases (15,45). Isolates of Pythium, Fusarium, Rhizoctonia, Phoma, and Gliocladium have been associated with this disease, but Fusarium spp. are the prevalent pathogens. Strains of these pathogens exhibited a wide range of virulence when tested on 'Midland' red clover (40,41). Infection is thought to occur in the seedling stage (41). Stands often fail to recover after the first cutting of the second year. The root and crown rot was more severe in years with high temperatures, limited soil moisture, and when the plants were grown under nutritional stress (8,17).

Pathogens associated with root and crown rot of alfalfa are Phytophthora (63), Aphanomyces (46,65), Pythium (65), Rhizoctonia (65), and Fusarium (1,13,65,70). Root and crown rot is aggravated by moisture and nutritional stress (10,55).

Nitrogen fixing bacteria, Rhizobium, are less effective in the presence of Fusarium spp. (38,45,53). The relationship between the bacteria and fungus is dependent upon soil pH, soil microflora, and time of inoculation (53). Plants with diseased roots have fewer nodules which may lead to a nitrogen stress and increased disease susceptibility.

Development of Selection Procedures

Techniques do not exist for selecting sainfoin plants with resistance to root and crown rot caused by Fusarium spp. After six years, only 3 of 100,000 plants were free of disease symptoms indicating that resistance is rare in existing populations (66). Therefore, selection procedures must be found that will screen large number of plants in short periods of time.

Early work on alfalfa root diseases was done on bacterial wilt, Corynebacterium insidiosum (McCull.) Jensen (14,39). Techniques developed to screen for resistance to this organism have been adapted to screen for other diseases caused by fungi such as Fusarium (13,14,21, 30,44,64). Techniques which appear favorable are inoculation of single roots, bare-root-soak, root-ball-soak, crown-soak, and inoculation of cut stems (13,14,45,63). The percentage of infected plants can be increased by wounding the roots prior to, or in conjunction with, inoculation (13,14,59,64). Because of the slow action of most root rotting pathogens, researchers visually score the spread of infection

three to six months after inoculation. This scoring system is usually based on five to seven categories and is used as the principal index of disease resistance (13,14,45).

It is possible to control the severity of a test by manipulating environmental factors. These factors include: age of plants at time of inoculation (2,14,47), water status (14,21), concentration of inoculum (14,45), the length of the trial period (14,21,47), and the temperature (14,21,45). These factors should be manipulated to create severe disease symptoms in inoculated sainfoin plants in a minimum amount of time. The development of the proper combination of these factors will require extensive investigation.

Chapter I

MATERIALS AND METHODS

General

The purpose of these experiments was to determine if: 1) the pathogens causing root and crown rot were seed-borne, 2) microconidia of F. solani could be used as inoculum, and 3) fungicides or seed treatments could reduce the severity of root and crown rot in sainfoin. Plants were grown either in the greenhouse or at the Field Research Laboratory, Bozeman, Montana. Plants in the greenhouse were grown with a 16-hour photoperiod provided by supplemental lighting from incandescent lights and were watered with a nutrient solution (Appendix Table 1). Seed and root pieces used for isolation were surface-sterilized by soaking in a 0.5% NaOCl solution for three minutes.

Data were analyzed by analysis of variance and means were separated by Duncan's New Multiple Range Test. Correlations were determined among indices within experiments.

Experiment 1: Determination of potentially pathogenic fungi associated with sainfoin seed

Samples of eight commercial seed lots of sainfoin were obtained from the Montana State Seed Testing Laboratory to determine if seed-borne pathogenic fungi were affecting sainfoin. The seed of each sample were divided into three groups and treated as follows:

Group I: Pod-intact - no treatment.

Group II: Pod-intact - surface-sterilized

Group III: Pod-removed - surface-sterilized

Eighty seed of each group were placed in petri dishes containing acidified potato dextrose sugar (HPDA)(66)(Appendix Table 1) and 80 seed were placed in petri dishes containing pentachloronitrobenzene agar (PCNBA)(57)(Appendix Table 1). HPDA was used for general fungal isolations and PCNBA was used to isolate Fusarium spp. The HPDA and PCNBA plates were incubated at 27 C for four and eight days, respectively. Fungal cultures were then identified by microscopic examination (72). The percentage of seeds contaminated with Alternaria spp. and/or F. oxysporum were analyzed by contingency chi square.

On the basis of laboratory isolations, two seed lots were chosen for further evaluation in the greenhouse. The seed pods were removed from one-half of the seed of both the seed lot heavily contaminated (#1538) and the seed lot lightly contaminated (#405) with Alternaria spp. One-half of both the 'pod-intact' and the 'pod-removed' seed were then surface-sterilized. One hundred seed from each of the four treatments of both seed lots were incubated for nine days at 25 C in germination trays to determine if treatments had affected germination. Treated seed were planted in Bozeman silt loam that had been either autoclaved at 121 C for six hours or pasturized for 16 hours with unpressurized steam. The experiment was conducted in a split-split-plot randomized complete block design with four

replications. The two seed lots were assigned to main plots, the two soil treatments to subplots, and the four seed treatments to sub-subplots. Each sub-subplot contained 50 seed.

After 24 days, seedling emergence was determined and the roots of four seedlings from each sub-subplot were harvested. A root piece was removed 1 cm below the crown of each seedling, surface-sterilized for 45 seconds, and placed on HPDA (66) to determine the percentage of seedlings infected with Alternaria spp.

Experiment II: An evaluation of the relative pathogenicity of microconidia and macroconidia of four isolates of F. solani

Three surface-sterilized, pod-removed 'Remont' seed were planted in 10.2 cm square plastic pots which contained Bozeman silt loam soil that had been autoclaved at 121 C for six hours. Seedlings were thinned to one per pot at emergence. Eighty-three days after emergence the seedlings were inoculated with F. solani by piercing the crown with a sterile dissecting needle and applying approximately 4×10^6 conidia to the wound.

The experiment was conducted in a split-plot randomized complete block design with three replications. Inoculation with either micro- or macroconidia was assigned to main plots. Microconidia were produced in Modified Eckert's Broth (66) and a mixture of micro- and macroconidia were produced in Snyder and Nash's sporulation medium (56)

Appendix Table 1). Inoculations with each of the four isolates of F. solani and with sterile water were assigned to subplots. There were 10 seedlings in each subplot.

Ninety days after inoculation, roots of seedlings were harvested, split longitudinally, and visually scored for disease severity. A single section of each taproot was removed 2 cm below the crown, surface-sterilized, and placed on HPDA (66) to determine the percentage of plants infected with F. solani.

Experiment III: Evaluation of soil fumigation and seed treatments for the control of root and crown rot of sainfoin.

A split-plot randomized complete block design with three replications was used to evaluate the effect of planting either pod-intact or pod-removed seed into pots which had been treated with fungicides. Surface-sterilized Remont seed with either the pod-intact or the pod-removed were randomly assigned to main plots. The fungicide treatments were assigned to subplots. These were:

Control: No treatment.

Chloropicrin Soil Fumigation: Chloropicrin (trichloronitromethane) was injected 10 cm into the soil in a 15.4 cm grid pattern with a Fumigun at the rate of 647 kg/ha. The treatment was applied on June 10, 1974, and the plots were sealed with plastic tarps for seven days.

Benlate Soil Drench: A 50% active-ingredient, wettable powder of the fungicide 'Benlate' (Methyl-(butylcarbanoyl)-2 benzimidazole carbamate) was incorporated 5 cm into the soil with a rake at the rate of 340 kg/ha. This treatment was applied on July 1, 1974.

Benlate Seed Treatment: Benlate was dusted on the seed at the rate of 2.5 g of wettable powder of the fungicide per 1000 g of seed immediately prior to planting.

The study was seeded at the Field Research Laboratory on July 9, 1974, at the rate of 24 and 34 kg/ha for the pod-removed and the pod-intact seed, respectively. Plots were 3.1 m long, 1.8 m wide, and contained 12 rows spaced 15.4 cm apart. The border surrounding each plot was 92 cm wide and seeded to crested wheatgrass. The plots were irrigated 20 minutes daily for 12 days to permit seedling establishment. Thereafter, irrigation water was applied as needed.

Five times during the course of the study a minimum of eight seedlings were removed from the margins of each plot and scored for nodulation (Table 1-1) and disease severity. A section of root from each plant was removed 3 cm below the crown, surface-sterilized, and placed on PCNBA (57) to determine the percentage of seedlings infected with Fusarium spp. Fusarium isolates were identified to species by cultural characteristics on slants of fresh potato dextrose agar (FPDA) (Appendix Table 1) and microscopic examination of macroconidia (72).

Table 1-1. The visual rating system used to assign nodulation scores to sainfoin seedlings.

Score	Description
1	No nodules formed
2	Nodules on 1% of the secondary roots
3	Nodules on 5% of the secondary roots
4	Nodules on 25% of the secondary roots
5	Nodules on 50% of the secondary roots
6	Nodules on 75% of the secondary roots

Forage yield, plant height, stand density, and seedling vigor were measured on a 1 m wide strip in the center of each plot. Table 1-2 lists the indices and the dates on which they were measured in this study.

RESULTS AND DISCUSSION

Experiment I: Determination of potential pathogenic fungi associated with sainfoin seed.

Helminthosporium, Nigrospora, Fusarium, Alternaria, and numerous saphrophytic fungi were found on sainfoin seed. Fifty-seven percent of the untreated seed were contaminated with Alternaria spp. (Table 1-3). Other fungal species occurred on less than five percent of the seed. Seed treatment reduced contamination with Alternaria spp. ($X^2 = 594.2$; $P < .005$). With the exception of one seed lot (#1538),

Table 1-2. The date of sampling and the indices measured on the soil fumigation and seed treatment experiment at the Field Research Laboratory, Bozeman, Montana.

Date		Index
1974	Aug. 26	Number of plants showing visual wilt symptoms
		Number of plants with visual root symptoms
		Percentage of plants infected with F. spp.
	Aug. 30	Stand density
		Seedling vigor scored
	Sept. 13	Number of plants with visual root rot symptoms
		Percentage of plants infected with F. oxysporum
		Percentage of plants infected with F. solani
	Oct. 8	Nodulation scored
		Percentage of plants infected with F. oxysporum
		Percentage of plants infected with F. solani
1975	June 5	Nodulation scored
		Disease severity scored
		Percentage of plants infected with F. oxysporum
		Percentage of plants infected with F. solani
	July 3	Forage yield
	Aug. 13	Plant height
	Sept. 29	Forage yield
	Oct. 8	Disease severity scored
		Percentage of plants infected with F. oxysporum
		Percentage of plants infected with F. solani

Table 1-3. The effect of pod removal and surface-sterilization of seed on the percentage of seed contaminated with either *Alternaria* spp. or *F. oxysporum* from eight commercial seed lots of sainfoin.

Seed lot	Contamination with *Alternaria* spp. 1/			Contamination with *F. oxysporum* 2/		
	PI 4/ Untreated	PI Surface 3/ sterilized	PR 5/ Surface sterilized	PI Untreated	PI Surface sterilized	PR Surface sterilized
	%	%	%	%	%	%
007	98.8	1.3	0.0	0.0	0.0	0.0
403	42.5	2.5	0.0	1.3	0.0	0.0
405	23.8	0.0	0.0	1.3	0.0	0.0
469	42.5	0.0	0.0	1.3	0.0	0.0
509	61.3	1.3	0.0	3.8	0.0	0.0
699	11.3	13.8	0.0	9.8	0.0	0.0
1005	75.0	0.0	0.0	0.0	0.0	0.0
1538	100.0	51.3	4.8	0.0	0.0	0.0

[1]Based on isolations made on HPDA (66).

[2]Based on isolations made on PCNBA agar (57).

[3]Seed surface sterilized by soaking in a 0.5% NaOCl solution for 3 minutes.

[4]Seed pod intact.

[5]Seed pod removal.

removing the pod and surface-sterilization of the seed eliminated this fungus (Table 1-3).

Two percent of the untreated seed were contaminated with F. oxysporum (Table 1-3). Surface-sterilization of the seed pod completely eliminated this fungus. Seed lots responded the same to seed treatments ($X^2 = 3.4$; $P > .995$). F. solani was never isolated from the pod or seed of sainfoin. This pathogen does not appear to be seed transmitted.

When planted in the greenhouse, seedling emergence of the seed lot heavily infested with Alternaria spp. (#1538) was reduced (Table 1-4). The degree of seedling infection was proportional to the amount of Alternaria spp. contamination on the seed. Since both seed lots had very similar germination in the laboratory, the reduction in emergence was probably due to seed rot or pre-emergence damping-off caused by Alternaria spp.

Seedling response was the same in both the pasteurized and autoclaved soils. Sears (66) reported sainfoin losses to Alternaria occurred only in heat treated soils. More information would have been gained if an untreated soil had been used as one of the soil treatments. Surface-sterilizing the pod, removing the pod, and surface-sterilizing the seed reduced the percentage of seedlings infected with Alternaria spp. but did not result in increased seedling emergence (Table 1-5).

Table 1-4. Sainfoin seedling emergence as affected by seed-borne Alternaria spp.

Seed lot	Seedling emergence 24 days %	Seedlings infected with Alternaria spp.[1] %	Seed germination laboratory[2] %
1538	68.8 a [3]	11.7 a	85.8
405	80.3 b	4.7 b	86.0

[1] Based on isolations made on HPDA (66).

[2] Means based on 400 seed.

[3] Means within a column not followed by the same letter differ at the .05 level of probability.

Table 1-5. The effect of pod removal and seed surface-sterilization on the percentage of seedlings infected with Alternaria spp. and percentage of seedling emergence.

Seed treatments	Seedling emergence 24 days %	Seedlings infected with Alternaria spp. [1] %
Pod-intact	79.6	18.8 a [3]
Pod-intact Surface-sterilized [2]	70.4	7.8 b
Pod-removed Surface-sterilized	76.2	1.6 b
Pod-removed	72.2	4.7 b

[1] Based on isolations made on HPDA (66).

[2] Surface-sterilized by soaking in a 0.5% NaOCl solution for three minutes.

[3] Means within the column not followed by the same letter differ at the .05 level of probability.

Experiment II: An evaluation of the relative pathogenicity of microconidia and macroconidia of four isolates of F. solani.

The microconidial inoculum infected a higher percentage of plants than did the macroconidial inoculum, but both inocula produced similar disease symptoms (Table 1-6). Both types of conidia are infectious, but the smaller microconidia may have more readily entered the wounds caused by the inoculation technique. The four isolates of F. solani produced different amounts of macroconidia in the Snyder and Nash's sporulation medium (56) (Table 1-7). The macroconidial inoculum was a composite of both microconidia and macroconidia, and probably did not accurately estimate the performance of an inoculum composed entirely of macroconidia.

Table 1-6. The effect of inoculating sainfoin seedlings with either microconidia or macroconidia of F. solani on the mean disease severity score and the percentage of plants infected.

Inoculum	Mean disease severity score [1]	Plants infected with F. solani [2]
Microconidia	2.05 a [3]	30.0 a
Macroconidia plus microconidia	1.87 a	21.6 b

[1] Disease severity scored as follows: 1 = no spread of discoloration; 2 = spread of discoloration less than 1 cm; 3 = spread of discoloration more than 1 cm; 4 = extensive vascular necrosis; and 5 = dead plant

[2] Based on isolations made on HPDA (66).

[3] Means within a column not followed by the same letter differ at the .05 level of probability.

The isolates caused similar disease symptoms and infected a similar number of seedlings (Table 1-7). Inoculation with any of the isolates resulted in more severe disease symptoms than inoculation with sterile water, but only one isolate infected a higher percentage of the seedlings than that observed in the control. Greater differences in the percentage of plants infected could probably have been detected had a more efficient inoculation procedure been used.

Table 1-7. The effect of inoculating sainfoin seedlings with four isolates of F. solani on the mean disease severity score and the percentage of plants infected. Also shown is the Percentage of microconidia produced in Snyder and Nash's sporulation medium (56).

Isolate	Mean disease severity score[1]	Plants infected with F. solani[2] %	Microconidia %
11	2.25 a [3]	16.7 ab	35
07	2.18 a	16.7 ab	50
06	2.17 a	18.3 a	5
01	1.98 a	11.7 ab	94
Control	1.20 b	1.7 b	0

[1] Disease severity scored as follows: 1 = no spread of discoloration; 2 = spread of discoloration less than 1 cm; 3 = spread of discoloration more than 1 cm; 4 = extensive vascular necrosis; and 5 = dead plant.

[2] Based on isolations made on HPDA (66).

[3] Means within a column not followed by the same letters differ at the .05 level of probability.

In future studies more resolution could be obtained by: 1) increasing the inoculum level from 4×10^6 to 4×10^8 conidia/plant; 2) using the root-cut-soak inoculation technique; 3) including the composited isolates as a treatment; 4) isolating the treatments to reduce contamination; and 5) developing procedures to obtain inoculum composed entirely of macroconidia.

Experiment III: Evaluation of soil fumigation and seed treatments for the control of the root and crown rot of sainfoin

Field fungicide treatments affected only the number of seedlings with wilt symptoms, seedling vigor, stand density, and plant height. The Benlate soil drench caused several emerging seedlings to wilt and reduced both stand density and seedling vigor (Table 1-8). This indicates that sainfoin is sensitive to high rates of Benlate. During the second year plant height differences were observed. Plants grown in the soil treated with Benlate and Chloropicrin were significantly taller than the control. This could be the initial expression of long-term effects. This study should be continued to determine other possible effects of the Benlate and Chloropicrin treatments.

No differences were detected in the isolation of Fusarium spp., disease severity, nodulation, or forage yield. This could have been the result of limited sample size, rapid invasion of the small plots by soil microbes, or lack of sufficient time for effects to be expressed. In future studies, the use of larger plots would allow the

Table 1-8. The effect of four fungicide treatments on sainfoin plants grown at the Field Research Laboratory, Bozeman, Montana.

Treatments	Number of wilted plants/plot	Stand density plants/meter row[1]	Mean seedling vigor score[2]	Plant height cm [3]
Chloropicrin soil fumigation	.50 a [4]	18.5 a	4.8 a	70.1 a
Benlate seed treatment	.33 a	16.3 ab	3.2 ab	67.6 ab
Control	.17 a	15.9 ab	3.2 ab	62.8 b
Benlate soil drench	2.17 b	13.8 b	1.7 b	69.3 a

[1] Means based on 10 samples of 1 m row/plot.

[2] Seedling vigor scored as follows: 1 = very poor growth; 2 = poor growth; 3 = fair growth; 4 = good growth; and 5 = excellent growth.

[3] Means based on 5 random samples per plot.

[4] Means within a column not followed by the same letters differ at the .05 level of probability.

sampling of more plants and reduce the possibility of invasion of microbes into treated plants.

CONCLUSIONS

Alternaria spp. are common contaminates of sainfoin seed. *F. oxysporum* contaminated the pod of two percent of the seed evaluated. *F. solani* was not found on the seed of sainfoin. The presence of potentially pathogenic fungi on sainfoin seed may require seed treatments to insure good seedling emergence in heat treated soils.

Both microconidia and macroconidia infected and caused disease symptoms in sainfoin. Microconidia were more effective in entering the small wounds caused by the inoculation technique. The four isolates were equally pathogenic.

The addition of fungicides to the seed and soil did not reduce the expression of the root and crown rot of sainfoin during the first 18 months of this study. Benlate fungicide was phytotoxic to a few seedlings indicating that sainfoin may be sensitive to this fungicide. This study should be monitored to detect possible long-term effects of the fungicide treatments.

Chapter II

MATERIALS AND METHODS

General

The purpose of these experiments was to determine: 1) the causal organism(s) responsible for root and crown rot of sainfoin, and 2) the host range of the organism(s). In all studies reported the following procedures were standard. Seed were surface-sterilized by soaking in a 0.5% NaOCl solution for three minutes. Three seeds were planted in each pot and seedlings were thinned to one per pot after emergence. Pots were 15.4 cm deep and 10.2 cm wide and contained a soil mixture of two parts Bozeman silt loam and one part river washed sand. The mixture had been autoclaved at 147 C for 85 minutes. Plants were grown with a 16-hour photoperiod provided by supplemental lighting from incandescent lights and were watered with a nutrient solution (Appendix 1).

Single spore cultures of *Fusarium* spp. were increased in Modified Eckert's Medium (66) in shake culture. Isolates of *F. solani* f. sp. *pisi* and *F. solani* f. sp. *phaseoli* were obtained from Washington State University (26). After inoculation, those plants inoculated with different pathogens were separated to prevent contamination. At the termination of each trial, roots were visually scored for disease severity. Two pieces of each root were removed 1 and 3 cm below the crown, surface-sterilized, and placed on HPDA (66) to determine the percentage of seedlings infected with *Fusarium* spp. Isolates of

Fusarium were identified to species on the basis of cultural characteristics on slant tubes of fresh potato dextrose agar (FPDA)(72)(Appendix 1). Disease severity scores and the percentage of plants infected with Fusarium were analyzed by analysis of variance. Duncan's New Multiple Range Test was used for mean separation.

Experiment I: A test of pathogenicity of F. solani and F. oxysporum on sainfoin.

The purpose of this test was to determine if F. solani and F. oxysporum were the pathogens responsible for the root and crown rot of sainfoin. Remont sainfoin seedlings were grown in a growth chamber with diurnal temperatures of 15.5 (night) and 25 C (day) for 83 days. Seedlings were then inoculated by severing their roots 5 cm below the crown, soaking the injured plants in inoculum for 15 minutes, and then transplanting the plants back into their pots. Plants were inoculated with either F. solani f. sp. pisi, F. solani f. sp. phaseoli, F. solani isolated from sainfoin, F. oxysporum isolated from sainfoin, or sterile water. A completely random design with five replications was used. Inocula of F. solani f. sp. pisi, F. solani 'sainfoin', and F. oxysporum 'sainfoin' contained 2.65×10^6, 2.70×10^6, and 22.50×10^6 microconidia/ml, respectively. The inoculum of F. solani f. sp. phaseoli contained mycelial mats instead of conidia. Ninety-five days after inoculation, roots were visually scored for disease severity and isolations made to determine the percentage of plants infected.

Experiment II: Evaluation of the host range of three isolates of F. solani isolated from beans, peas, and sainfoin.

The purpose of this experiment was to determine if the three isolates of F. solani had common host ranges. 'Ladak 65' alfalfa and Remont sainfoin seedlings were 65 days old and the 'Alaskan' pea and red kidney bean seedlings were 28 days old at the time of inoculation. Seedlings were inoculated by piercing the hypocotyl with a sterile dissecting needle and applying 2.0×10^8 microconidia to the wound. Twelve plants of each species were inoculated with either F. solani f. sp. pisi, F. solani f. sp. phaseoli, F. solani 'sainfoin', or sterile water. The plants were arranged in a split-plot randomized complete block design with three replications. The four pathogen treatments were assigned to main plots and the four host species were assigned to subplots. Each subplot contained four seedlings. Thirty-two days after inoculation, roots were visually scored for disease severity and isolations were made to determine the percentage of plants infected. A pooled error term was used to compute the mean separation (71).

RESULTS AND DISCUSSION

Experiment I: A test of the pathogenicity of F. solani and F. oxysporum on sainfoin.

The isolate of F. oxysporum infected sainfoin but produced only sporatic vascular discoloration of limited severity (Table 2-1). This

limited symptom development indicates that F. oxysporum is not responsible for the root and crown rot of sainfoin.

Table 2-1. The pathogenicity of F. oxysporum and F. solani on sainfoin seedlings as measured by the mean disease severity score and the percentage of plants infected.

Pathogens	Mean disease severity score [1]	Percentage of plants infected [2]
Control	1.4 a [4]	0
F. oxysporum 'sainfoin' [3]	2.0 a	100
F. solani f. sp. phaseoli	4.2 b	100
F. solani 'sainfoin'	4.2 b	100
F. solani f. sp. pisi	4.4 b	100

[1] Disease severity scored as follows: 1 = no spread of discoloration; 2 = spread of discoloration less than 1 cm; 3 = spread of discoloration more than 1 cm; 4 = extensive vascular necrosis; and 5 = dead plant.

[2] Based on isolations made on HPDA (66).

[3] Isolated from diseased sainfoin plants.

[4] Means within a column not followed by the same letter differ at the .05 level of probability.

The three isolates of F. solani infected sainfoin and produced disease symptoms of equal severity (Table 2-1). Inoculated plants had extensive vascular necrosis very similar to disease symptoms observed in naturally infected sainfoin plants. These results provide evidence that F. solani is responsible for the root and crown rot of sainfoin.

The tests with F. solani f. sp. pisi and F. solani f. sp. phaseoli indicate that sainfoin may be susceptible to many isolates of this organism. Because peas, beans, and other legumes have been grown for many years in Montana, it is possible that isolates of F. solani have spread and proliferated in agricultural lands. This could explain the rapid development of root and crown rot of sainfoin in areas where this crop has never been grown.

Experiment II: Evaluation of the host range of three isolates of F. solani isolated from beans, peas, and sainfoin.

The three isolates of F. solani were equally pathogenic on sainfoin and beans (Table 2-2). F. solani 'sainfoin' was not highly pathogenic to peas and was more pathogenic of alfalfa than F. solani f. sp. phaseoli. F. solani f. sp. pisi was pathogenic on all hosts tested. Crop rotations which include beans, peas, or alfalfa would not control sainfoin root and crown rot. Stands of sainfoin established in fields with a recent history of these crops would be more rapidly attacked by root and crown rot than stands established in areas without a prior history of these crops.

The bean plants inoculated with sterile water were discolored and infected with F. solani (Table 2-2). This was probably due to the slow formation of callus tissue by bean seedlings following inoculation with the sterile dissecting needle. Controls of other species had a lower percentage of infection.

Table 2-2. The pathogenicity of three isolates of F. solani on peas, beans, alfalfa, and sainfoin as measured by the mean disease severity score and the percentage of plants infected.

Host	Pathogen	Mean disease severity score[1]	Plants infected with F. solani[2] %
Peas:			
	F. solani f. sp. pisi	5.00 a[4]	100.0 a
	F. solani f. sp. phaseoli	4.33 ab	83.3 abc
	F. solani 'sainfoin'[3]	2.50 fg	8.3 e
	Control	1.42 h	0.0 e
Beans:			
	F. solani f. sp. pisi	3.42 cde	91.8 ab
	F. solani f. sp. phaseoli	3.25 cdef	100.0 a
	F. solani 'sainfoin'	2.92 def	100.0 a
	Control	2.42 f	41.8 d
Alfalfa:			
	F. solani 'sainfoin'	3.58 bcd	83.3 abc
	F. solani f. sp. pisi	3.08 cdef	100.0 a
	F. solani f. sp. phaseoli	2.67 ef	83.3 abc
	Control	1.33 h	8.3 e
Sainfoin:			
	F. solani 'sainfoin'	3.83 bc	67.7 bc
	F. solani f. sp. phaseoli	3.42 cde	58.3 cd
	F. solani f. sp. pisi	3.25 cdef	75.0 abc
	Control	1.67 gh	8.3 e

[1] Disease severity scored on spread of discoloration from point of inoculation as follows: 1 = 0.1 - 0.9 cm; 2 = 1.0 - 1.9 cm; 3 = 2.0 - 2.9 cm; 4 = 3.0 - 5.0 cm or extensive vascular necrosis; 5 = dead plant.

[2] Based on isolations made on HPDA (66).

[3] Isolated from diseased sainfoin plants.

[4] Means within a column not followed by the same letters differ at the .05 level of probability.

The lack of specificity of the isolates evaluated in this study question the concept of 'formae speciales' in fungi such as F. solani. It is possible that this lack of specificity is an artifact of injecting pathogens into host species and that many of these isolates would not be pathogenic under natural conditions. Further studies are needed to test this concept and to define the host range of F. solani 'sainfoin'. These studies should include a wide range of native and cultivated legumes.

CONCLUSIONS

F. solani f. sp. pisi, F. solani f. sp. phaseoli, and F. solani 'sainfoin' caused root and crown rot in sainfoin. F. oxysporum did not appear to be responsible for root and crown rot.

Chapter III

MATERIALS AND METHODS

General

The purpose of these studies was to develop and initiate screening procedures to detect potential sources of resistance to root and crown rot of sainfoin caused by F. solani. All plants were grown either in the greenhouse or at the Field Research Laboratory, Bozeman, Montana. Plants in the greenhouse were grown with a 16-hour photoperiod provided by supplemental lighting from incandescent lights and were watered with a nutrient solution (Appendix Table 1). Root pieces used for isolation and seed were surface-sterilized by soaking in a 0.5% NaOCl solution for three minutes.

All data were analyzed by analysis of variance. Harvey's (29) least squares analysis was used when there were unequal numbers in each replication. Means were separated by Duncan's New Multiple Range Test. Mean separations were computed with a pooled error term in those experiments with multiple error terms (71). Correlations were determined for the indices within experiments.

Experiment I: Greenhouse evaluation of four techniques of inoculating sainfoin seedlings with F. solani.

Two- and six-week-old sainfoin seedlings were inoculated with either F. solani or sterile water using four techniques. The experiment was conducted in a split-split-plot randomized complete block design with four replications. Seedling ages were randomly assigned to

main plots; inoculation with F. solani or sterile water to subplots; and the four techniques of inoculation to sub-subplots. Within each age group, one half of the seedlings were inoculated with sterile water instead of F. solani to allow an assessment of the physical injury resulting from the inoculation techniques. Each inoculation technique was applied to 10 seedlings in each sub-subplot. Techniques were:

Root-Cut-Soak: Seedlings were removed from the pots and the roots severed 5 cm below the crown. They were then soaked in inoculum for 15 minutes and transplanted back into the pots.

Crown Injection: The crowns were pierced with a sterile dissecting needle and 5 ml of inoculum applied to the wound.

Aerial Spray: Seedlings were sprayed with inoculum at 14 p.s.i. The foliage was removed 5 cm above the crown of the six-week-old seedlings prior to inoculation, to increase penetration of the inoculum into the crown area.

Toothpick: Wooden toothpicks were placed in 20 cm test tubes, soaked in Modified Eckert's Broth (66), autoclaved at 154 C for 15 minutes, and then incubated with F. solani for 14 days. These toothpicks were pierced through the seedling's crowns where they remained until the termination of the study. Sterile toothpicks were used as the control. This

technique was not used on two-week-old seedlings because their crowns were too small.

Three Remont seed were planted in each pot and seedlings were thinned to one per pot after emergence. The plastic pots were 10.2 cm square and contained a Bozeman silt loam soil that had been autoclaved for six hours at 97 C.

The inoculum was a composite of four isolates of F. solani isolated from sainfoin at different Montana locations. Each isolate was increased individually on Modified Eckert's Broth (66) in shake culture and equal proportions of conidia were combined prior to inoculation. The composited inoculum contained 9.1×10^7 microconidia/ml.

Ninety days after inoculation, counts were made to determine seedling mortality. The roots were then harvested, split longitudinally, and visually scored for disease severity (Figure 3-1). A section of each root was removed from the crown, surface-sterilized, and placed on PCNBA (57) to determine the percentage of plants infected with F. solani.

The mean disease severity score and the percentage of plants infected were used as indices of inoculation effectiveness. Data obtained from inoculating six-week-old seedlings were analyzed separately in a split-plot randomized complete block design to evaluate the toothpick inoculation technique. The toothpick data were omitted when the indices were analyzed over both dates of inoculation.

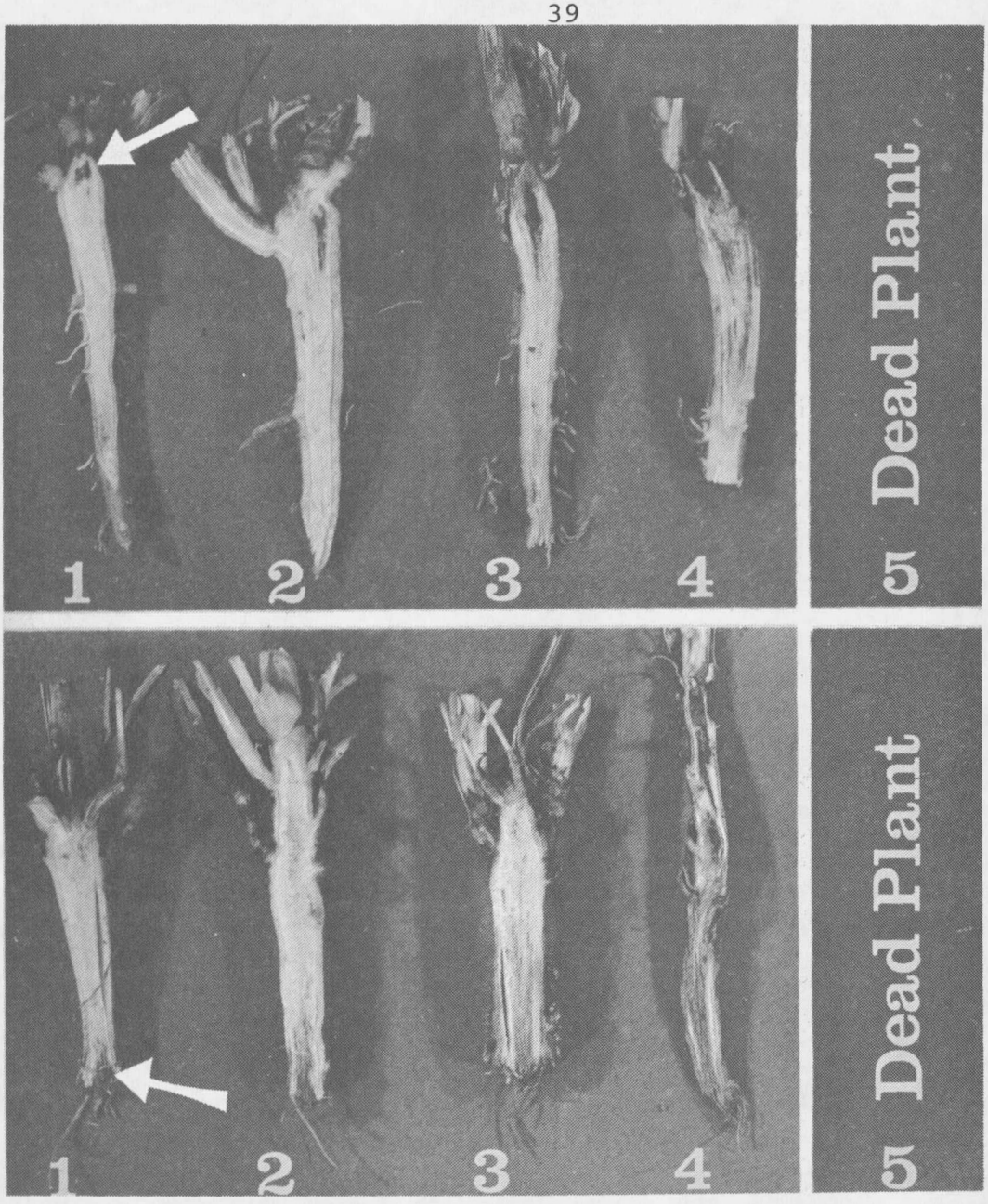

Figure 3-1: The visual scoring system used to assign disease severity scores to the aerial spray, crown injection, and toothpick techniques is shown above and the root-cut-soak technique is shown below. Arrows denote point of inoculation. Disease severity scored as follows: 1 = no spread of discoloration; 2 = spread of discoloration less than 1 cm; 3 = spread of discoloration more than 1 cm; 4 = extensive vascular necrosis; and 5 = dead plant.

Experiment II: Field evaluation of four techniques of inoculating sainfoin seedlings with F. solani.

Remont seed were planted in the field in rows 60 cm apart and 12 m long. Sixty days after emergence, seedlings were thinned to one plant every 15 cm. Plants were irrigated as needed.

The experiment was conducted in a split-plot randomized complete block design with four replications. Inoculation treatments, either with F. solani or sterile water, were assigned to mainplots; and the four previously described inoculation techniques were assigned to subplots. A minimum of 25 seedlings in each subplot were inoculated 83 days after emergence. The composited inoculum contained 11.3×10^7 microconidia/ml.

One year after inoculation, plants were counted to determine mortality. Roots were scored for disease severity and the percentage of plants infected with F. solani determined by isolations on PCNBA (57).

Experiment III: An evaluation of the repeatibility and resolution of the root-cut-soak technique of inoculating replicated genotypes of sainfoin.

Vegetative cuttings were made of 10 sainfoin clones that had been selected on the basis of being either solid crowned (SC) or free of visual disease symptoms (DR). Crown buds of each clone were placed in the mist bench for 45 days. Those buds which initiated root growth were transplanted into plastic pots 15.4 cm deep and 10.2 cm wide.

Pots contained a soil mixture of perlite, washed sand, and Bozeman silt loam. These cuttings were grown in the greenhouse until they had flowered. The foliage was then removed and they were inoculated with F. solani using the root-cut-soak inoculation technique. The composited inoculum contained 3.0×10^7 microconidia/ml.

One hundred and four days after inoculation, the plant roots were harvested and scored for disease severity (Figure 3-1). Two sections of each root were removed 1 and 3 cm below the crown, surface-sterilized, and placed on HPDA (66) to determine the percentage of cuttings infected with F. solani.

Experiment IV: An evaluation of the disease resistance of the open-pollinated progeny of 31 clones of sainfoin.

Open-pollinated seed of 31 clones of sainfoin which had been selected for longevity under field conditions was tested for resistance to root and crown rot caused by F. solani. Melrose, Eski, and Remont were check cultivars. Also included in the study were: an experimental cultivar developed in New Mexico; open-pollinated seed from a 12-year-old stand of Eski near Creston (Population I); and open-pollinated seed from an eight-year-old stand near Bozeman (Population II).

Ninety seeds of each entry were planted into greenhouse benches containing plaster-grade vermiculite. Seedlings were inoculated 42 days after emergence with F. solani using the root-cut-soak

inoculation technique. They were then transplanted into benches containing an untreated Bozeman silt loam soil in rows 9 cm apart with plants 7 cm apart within rows. The foliage was removed 5 cm above the crown after transplanting to increase uptake of the inoculum. The composited inoculum contained 8.8×10^7 microconidia/ml. Each entry was represented by 10 seedlings in each of the three replications arranged in a randomized complete block design.

Twenty-four days after inoculation, counts were made to determine seedling mortality. Dead plants were considered to have died from transplant shock and were not considered in the disease evaluation. Ninety days after inoculation, roots were scored for disease severity (Figure 3-1) and the range, variance, and mean for each entry was calculated. Isolations from each plant were made on PCNBA (57) to determine the percentage of plants infected with F. solani. The mean disease severity score and the percentage of plants infected were used as indices of disease susceptibility.

Experiment V: Screening 296 accessions of the world collection of Onobrychis for sources of resistance to crown and root rot caused by F. solani.

Seed of 296 accessions of Onobrychis obtained from the USDA Plant Materials Center, Pullman, Washington, were planted into greenhouse benches filled with plaster-grade vermiculite. The seedlings were inoculated with F. solani 105 days after emergence using the root-cut-soak inoculation technique. The composited inoculum

contained 7.3×10^7 microconidia/ml. The seedlings were then transplanted into greenhouse benches as previously described. The foliage was removed from the seedlings 8 cm above the crown.

Those accessions with less than 15 healthy seedlings were evaluated in an unreplicated trial in which each accession was represented by eight seedlings. Accessions with 15 or more healthy seedlings were evaluated in a randomized complete block design with three replications. Seedling mortality was determined 16 days after inoculation. Dead seedlings were assumed to have died from transplant shock and were not considered in the disease evaluation.

The unreplicated trial was terminated 89 days after inoculation. Replications I, II, and III were harvested 110, 112, and 123 days after inoculation, respectively. At time of harvest, seedlings were scored for disease severity and a random sample of 50 root pieces from each replication were surface-sterilized and placed on HPDA (66) to determine the percentage of plants infected with F. solani. In replications II and III an expanded scale was used to assign disease severity scores. Scores of 3-, 3, and 3+ were assigned numeric values of 2.5, 3.0, and 3.5, respectively. This expanded scale was necessary to differentiate among the broad range of symptoms within the 3 category. The range, variance, and mean disease severity scores were calculated for each accession.

RESULTS AND DISCUSSION

Experiment I: Greenhouse evaluation of four techniques of inoculating sainfoin seedlings with F. solani.

The inoculation techniques differentially affected the percentage of sainfoin plants infected with F. solani, the severity of disease symptoms, and seedling mortality (Table 3-1). An effective inoculation technique should cause a high percentage of infection, produce severe disease symptoms, and result in a low seedling mortality from physical injury. The aerial spray on two-week-old and six-week-old seedlings, and the crown injection on two-week-old seedlings failed to meet these criteria (Figures 3-2 and 3-3). The crown injection and toothpick techniques on six-week-old seedlings produced moderate disease symptoms, but infected only 58 and 48 percent of the seedlings, respectively.

Inoculation of two-week-old and six-week-old sainfoin seedlings with the root-cut-soak technique resulted in 77 percent of the plants becoming infected with F. solani (Table 3-1). However, the six-week-old seedlings had the most severe disease symptoms and the lowest seedling mortality. For these reasons, the root-cut-soak technique applied to six-week-old seedlings were selected as the best mass screening procedure.

The mean disease severity score and the percentage of plants infected with F. solani were highly correlated ($r = .86$ **). This

Table 3-1. The effect of four inoculation techniques on the disease severity score, the percentage of plants infected with F. solani, and mortality of sainfoin seedlings inoculated in the greenhouse.

Age of seedlings & inoculation technique	Disease severity score [1]	Plants infected with F. solani %	Seedling mortality %
Inoculation with F. solani			
2 weeks:			
Root-cut-soak	2.36 b [3]	78.3 a	63
Crown injection	1.92 cd	19.5 c	8
Aerial spray	1.43 de	10.0 e	0
6 weeks:			
Root-cut-soak	3.13 a	77.5 a	10
Crown injection	2.58 b	57.5 b	0
Toothpick	2.73	47.5	0
Aerial spray	1.28 ef	20.0 c	0
Inoculation with sterile H_2O			
2 weeks:			
Root-cut-soak	1.67 cd	6.0 f	50
Crown injection	1.42 de	12.5 d	5
Aerial spray	1.08 f	5.0 f	3
6 weeks:			
Root-cut-soak	1.73 cd	10.0 e	0
Crown injection	1.38 de	2.5 g	0
Toothpick	1.56	5.0	0
Aerial spray	1.18 ef	0.0 h	0

[1] Disease severity scored as follows: 1 = no spread of discoloration; 2 = spread of discoloration less than 1 cm; 3 = spread of discoloration more than 1 cm; 4 = extensive vascular necrosis; and 5 = dead plant.

[2] Based on isolations made on PCNBA (57).

[3] Means with a column not followed by the same letter differ at the .05 level of probability.

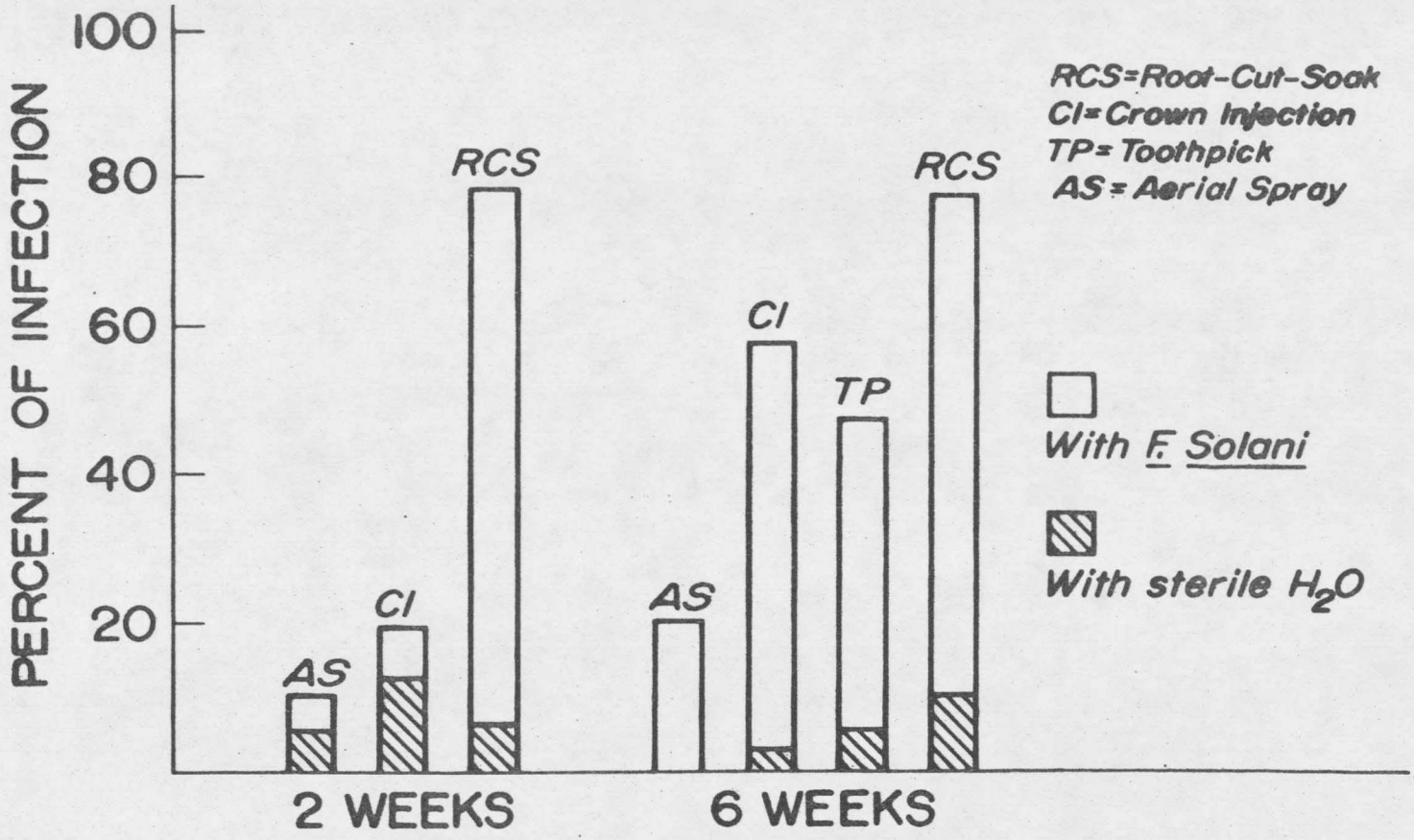

Figure 3-2. The effect of four inoculation techniques on the percentage of plants infected with F. solani when inoculated two weeks and six weeks after emergence. Isolations were made on PCNBA (57).

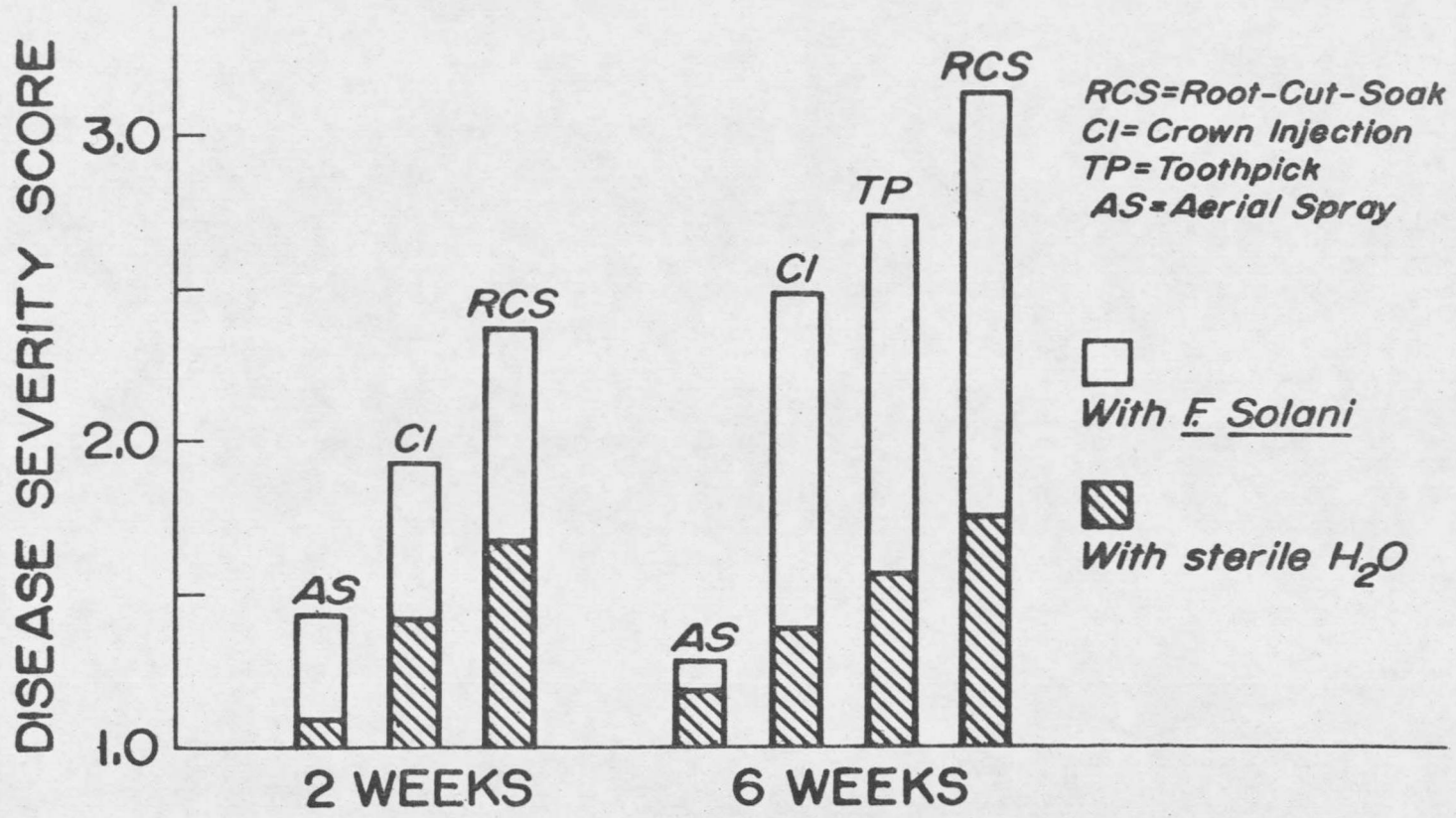

Figure 3-3. The effect of four inoculation techniques on the mean disease severity score assigned to sainfoin seedlings inoculated with F. solani at two weeks and six weeks of age. Disease severity scored as follows: 1 = no spread of discoloration; 2 = spread of discoloration less than 1 cm; 3 = spread of discoloration more than 1 cm; 4 = extensive vascular necrosis; and 5 = dead plant.

strong association indicates that plants could be screened on the single index of the mean disease severity score.

The coefficient of variability of the percentage of plants infected with F. solani was high (CV = 45.3%). This index was based on isolations from a single root piece from each plant. In future studies, this index should be based on multiple root pieces to minimize this source of variability.

Experiment II: Field evaluation of four techniques of inoculating sainfoin seedlings with F. solani.

The inoculation techniques differentially affected both the mean disease severity score and the percentage of plants infected with F. solani. The physical injury resulting from the inoculation techniques was responsible for these differences since inoculation with both F. solani and sterile water produced similar disease expression (Figures 3-4 and 3-5). This indicates that either the natural inoculum was at a high level or that microconidia of F. solani are ineffective as inoculum under field conditions.

The root-cut-soak technique produced the most severe disease symptoms and caused the highest percentage of infection (Figures 3-4 and 3-5). This technique, however, was so severe that 75 percent of the seedlings inoculated died during the year-long trial period (Table 3-2). Seedling mortality may have resulted from physical injury

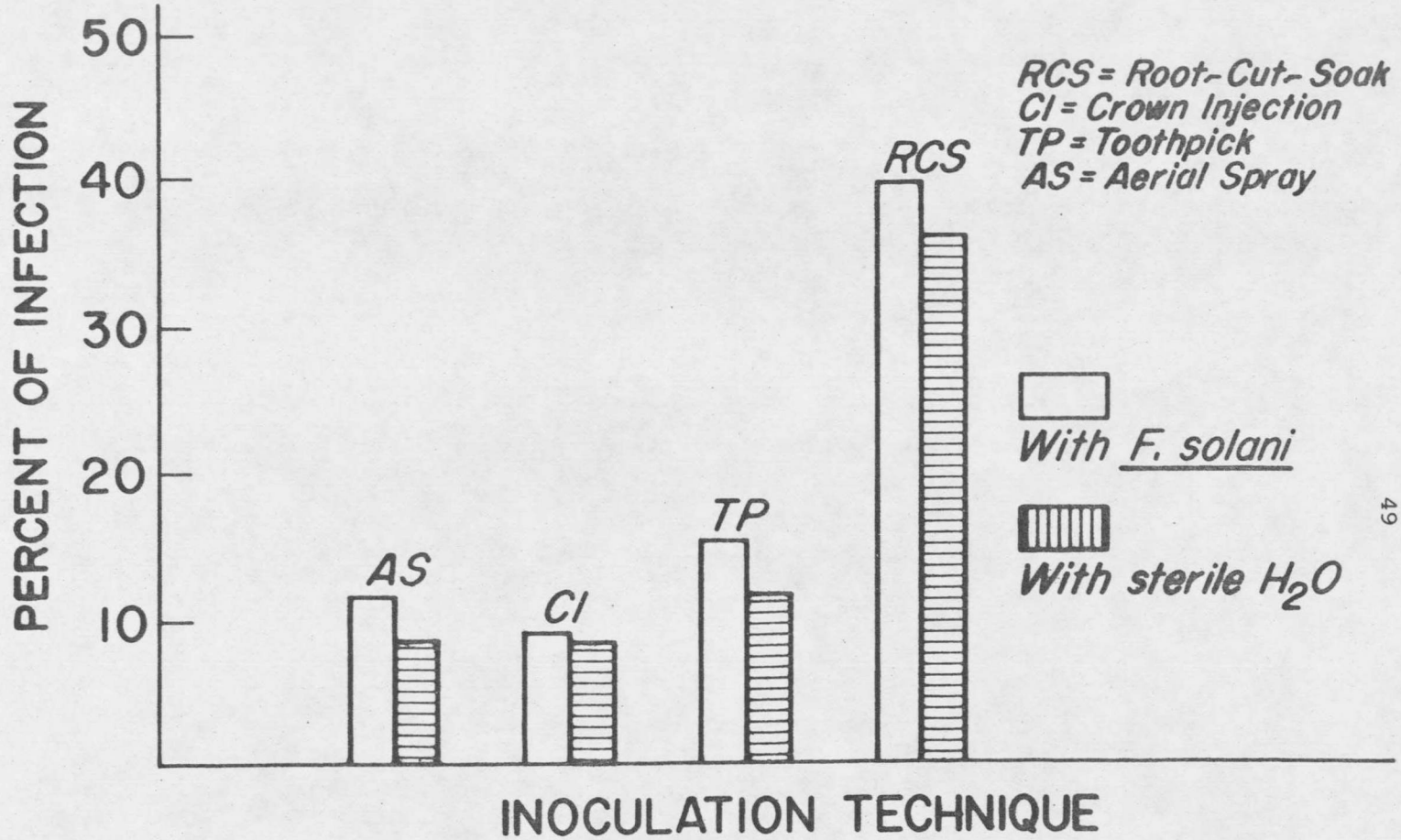

Figure 3-4. The effect of four inoculation techniques on the percentage of plants infected with F. solani when inoculated 83 days after emergence at the Field Research Laboratory, Bozeman, Montana. Isolations were made on PCNBA (57).

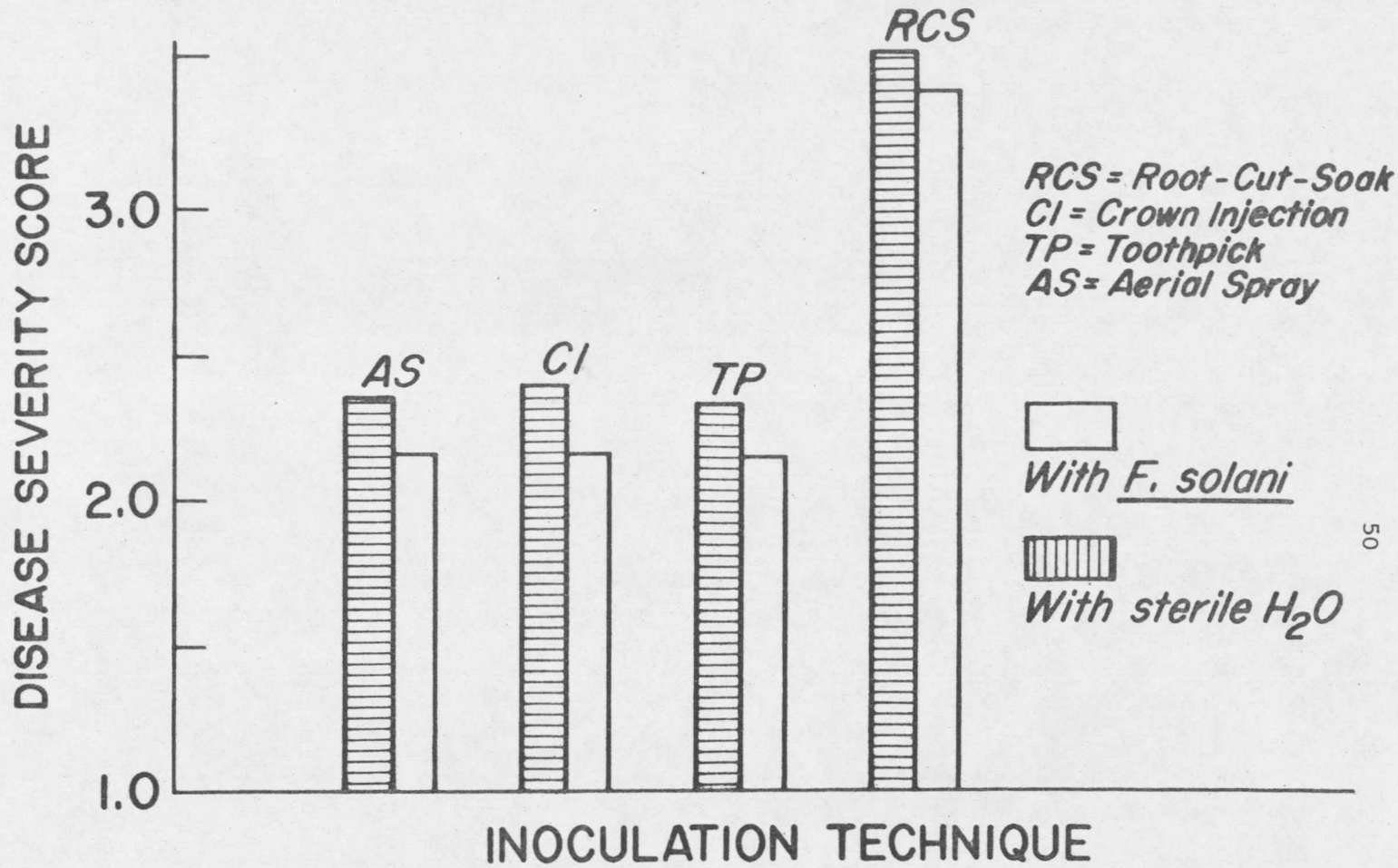

Figure 3-5. The effect of four inoculation techniques on the mean disease severity score assigned to sainfoin seedlings inoculated with F. solani at the Field Research Center, Bozeman, Montana. Disease severity scored as follows: 1 = no spread of discoloration; 2 = spread of discoloration less than 1 cm; 3 = spread of discoloration more than 1 cm; 4 = extensive vascular necrosis; and 5 = dead plant.

Table 3-2. The effect of four inoculation techniques on the disease severity score, the percentage of plants infected with F. solani, and mortality of sainfoin seedlings inoculated 83 days after emergence at the Field Research Laboratory, Bozeman, Montana.

Inoculation technique	Disease severity score [1]	Plants infected with F. solani [2] %	Seedling Mortality %
Inoculation with F. solani			
Root-cut-soak	3.40 a [3]	39.5 a	79
Crown injection	2.37 b	9.1 b	26
Toothpick	2.33 b	14.6 b	17
Aerial spray	2.21 b	10.6 b	2
Inoculation with sterile H_2O			
Root-cut-soak	3.31 a	35.0 a	75
Crown injection	2.21 b	8.8 b	15
Toothpick	2.20 b	11.3 b	20
Aerial spray	2.36 b	8.9 b	11

[1] Disease severity scored as follows: 1 = no spread of discoloration; 2 = spread of discoloration less than 1 cm; 3 = spread of discoloration more than 1 cm; 4 = extensive vascular necrosis; and 5 = dead plant.

[2] Based on isolations made on PCNBA (57).

[3] Means within a column not followed by the same letter differ at the .05 level of probability.

rather than from disease. This technique has potential for mass screening but must be refined to reduce seedling mortality.

The mean disease severity score and the percentage of plants infected with F. solani were highly correlated (r = .98 **). Thus, mass screening in the field could be based on the single index of disease severity without direct isolation from every plant.

The percentage of plants infected with F. solani was much lower in the field than in the greenhouse. This could be due to the age of plants at time of inoculation. Plants in the field were 41 days older than those in the greenhouse at time of inoculation. Further investigations are needed to refine field inoculation techniques before screening is initiated.

Experiment III: An evaluation of the repeatibility and resolution of the root-cut-soak technique of inoculating replicated genotypes of sainfoin with F. solani.

An effective inoculation technique applied to cuttings of the same clone should produce disease symptoms of a constant severity. This would produce disease severity scores with a narrow range and a low variance which permits detection of genotypic differences among clones. Except for three clones, the range was within plus or minus one scoring unit of the mean (Table 3-3). The homogeneous variances ranged from .20 to 1.00. Thus, the disease severity scores assigned to cuttings of the same clone were repeatable. Both the range and

Table 3-3. The response of the vegetative cuttings of 10 sainfoin clones inoculated with F. solani using the root-cut-soak technique.

Clone	Cuttings evaluated	Mean	Variance	Range
SC 8	5	2.60 a [2/]	.30	2 - 3
DR 6	3	2.67 ab	.33	2 - 3
SC 10	3	3.00 abc	1.00	2 - 4
DR 2	3	3.33 abcd	.36	3 - 4
SC 11	5	3.60 abcd	.30	3 - 4
SC 3	4	3.75 bcd	.92	3 - 5
DR 5	5	3.80 cd	.70	3 - 5
SC 7	5	3.80 cd	.20	3 - 4
SC 4	7	4.00 cd	.67	3 - 5
SC 9	8	4.25 cd	.50	3 - 5

[1] Disease severity scored as follows: 1 = no spread of discoloration; 2 = spread of discoloration less than 1 cm; 3 = spread of discoloration more than 1 cm; 4 = extensive vascular necrosis; and 5 = dead plant

[2] Means not followed by the same letter differ at the .05 level of probability.

variance might be reduced if the visual scoring system (Figure 3-1) were expanded from five to seven categories. Such an expansion would require a longer trial period to increase the severity of disease symptoms and a more rigid scoring index.

The clones had different mean disease severity scores. Only those differences of one scoring unit or greater were significant (Table 3-3). This limited resolution was due to small sample sizes. Resolution might be improved by increasing the number of scoring categories, increasing the length of the trial period, and increasing the number of plants evaluated.

The root-cut-soak technique was effective. F. solani was isolated from 95 percent of the cuttings and obtained an evaluation of each clone's disease susceptibility. Current breeding material and other sources of germplasm should be evaluated for potential sources of disease resistance using the root-cut-soak inoculation technique.

Experiment IV: An evaluation of the disease resistance of the open-pollinated progeny of 31 clones of sainfoin.

Disease severity scores differed significantly among entries. Scores ranged from 2.82 to 4.22 (Appendix Table 2) and their distribution approximated a normal curve (Figure 3-6). This indicates that genetic differences do exist among the entires in their resistance to

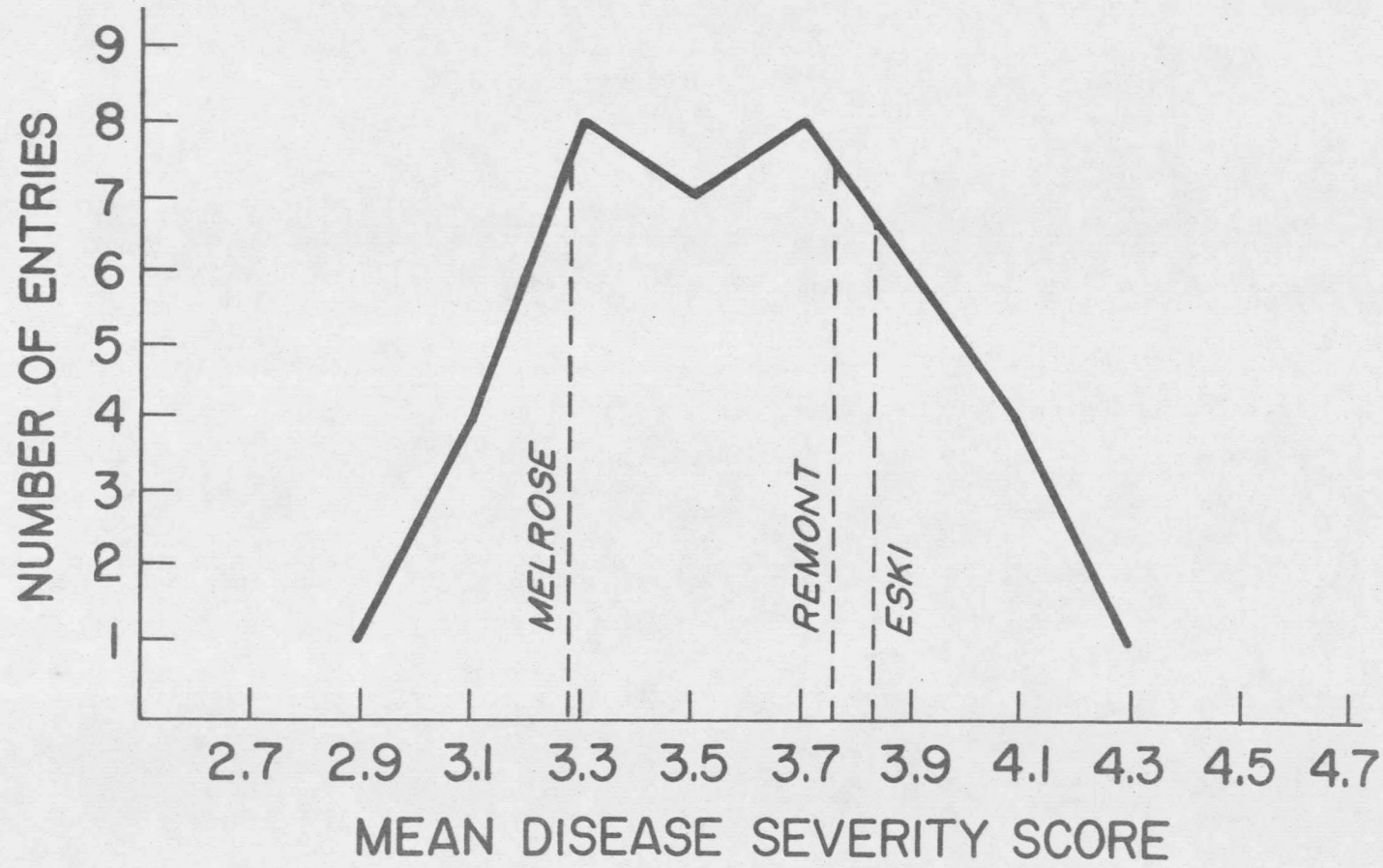

Figure 3-6. The response of the open-pollinated progeny of 39 entries to inoculation with F. solani as measured by the mean disease severity score of the entries. Disease severity scored as follows: 1 = no spread of discoloration; 2 = spread of discoloration less than 1 cm; 3 = spread of discoloration more than 1 cm; 4 = extensive vascular necrosis; and 5 = dead plant.

root and crown rot caused by F. solani. Selection within this population should increase disease resistance.

Population I, which had undergone 12 years of natural selection, was not significantly better than its parental variety, Eski (Appendix Table 2). Population II and many of the clones which had been selected for longevity in the field were very disease susceptible. Natural selection appears to be ineffective in developing resistance to the inoculated pathogen. It is possible that naturally selected populations would be attacked by F. solani less under field conditions due to the development of morphological barriers which prevent infection. The development of true physiological resistance probably could be accomplished by screening with the root-cut-soak inoculation technique.

Melrose was the most disease resistant cultivar evaluated and may have some resistance to F. solani (Figure 3-6). It is possible that selection could be practiced within this and other existing cultivars as demonstrated by the wide range and large variance in their disease severity score (Appendix Table 2).

Twenty-four days after inoculation, 45 percent of the seedlings had died because of severe transplant shock. In future studies, mortality could be reduced by inoculating older seedlings, removing the foliage at 8 cm instead of 5 cm above the crown, and by pasturizing the soil benches prior to transplanting.

Experiment V: Screening 296 accessions of the world collection of Onobrychis for sources of resistance to crown and root rot caused by F. solani.

The 11 species of Onobrychis and the accessions within the species of tanaitica, transcaucasica, arenaria, sibirica, and viciifolia differed in disease susceptibility (Table 3-4). Mean disease severity scores of the accessions ranged from 2.79 to 4.62 and their distribution closely approximated a normal curve (Figure 3-7). Most accessions had disease severity scores with a wide range and a large variance (Appendix Table 3). Thus, one could select for disease resistance among species, among accessions within a species, or among plants within an accession. Most rapid progress could be obtained by selecting the best plants within the best accessions of the most disease resistant species.

The mean disease severity scores of the check cultivars, Melrose and Remont were similar to the scores observed in Experiment IV. Eski's mean disease severity score was .48 scoring units lower in this trial than in the previous trial (Table 3-5). This disparity may have been due to the small sample used in the earlier experiment or because different seed lots of Eski were used in the trials. In future studies, seed of check cultivars should be taken from the same seed lot.

The mean disease severity scores of the 113 accessions of Onobrychis evaluated in the unreplicated trial ranged from 2.88 to

5.00 (Appendix Table 4). Values presented were based on an evaluation of eight or fewer plants, and should be used only as an initial evaluation.

Table 3-4. The response of 11 species of Onobrychis to inoculation with F. solani as measured by the mean disease severity score.

Species	Mean disease severity score [1]	Accessions Evaluated	Plants evaluated
O. tanaitica	3.31 a [2]	14	233
O. vulgaris	3.32 ab	8	124
O. iberica	3.38 abc	2	30
O. inermis	3.47 abc	14	273
Check Cultivars [3]	3.49 abc	3	60
O. transcaucasica	3.51 bc	70	1187
O. biebersteinii	3.61 bcd	3	50
O. antasiatica	3.61 bcd	2	40
O. arenaria	3.64 cd	38	704
O. altissima	3.64 cd	4	66
O. sibirica	3.65 cd	2	36
O. viciifolia	3.80 d	24	383

[1] Disease severity scored on spread of discoloration from point of inoculation as follows: 1 = no spread; 2 = 0.1 - 0.9 cm; 2.5 = 1.0 - 1.9 cm; 3 = 2.0 - 2.9 cm; 3.5 = 3.0 - 3.9 cm; 4 = extensive vascular necrosis; and 5 = dead plant.

[2] Means not followed by the same letters differ at the .05 level of probability.

[3] Check cultivars consisted of Eski, Remont, and Melrose.

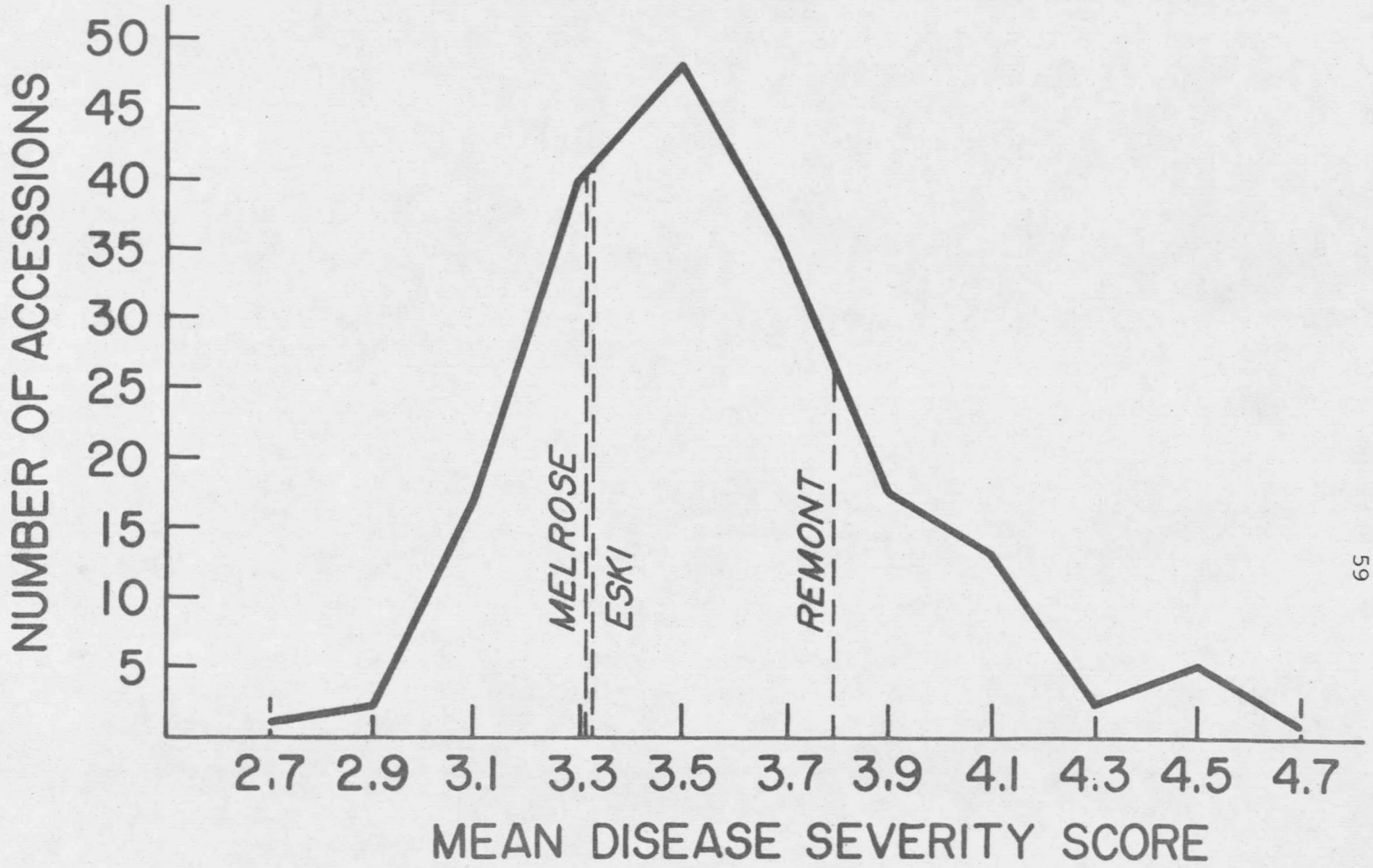

Figure 3-7. The response of 181 accessions of the world collection of Onobrychis to inoculation with F. solani as measured by the mean disease severity score of each accession. Disease severity scored on spread of discoloration from point of inoculation as follows: 1 = no spread; 2 = 0.1 - 0.9 cm; 2.5 = 1.0 - 1.9 cm; 3 = 2.0 - 2.9 cm; 3.5 = 3.0 - 3.9 cm; 4 = extensive vascular necrosis; and 5 = dead plant.

Table 3-5. The response of three cultivars of sainfoin to inoculation with F. solani in two trials as measured by the mean, variance and range of the disease severity scores.

Cultivar	Experiment IV				Experiment V			
	Number of plants	Disease Severity Score [1]			Number of plants	Disease severity score [2]		
		Mean	Variance	Range		Mean	Variance	Range
Melrose	24	3.29	.65	2 - 5	18	3.31	.80	2.5-5
Eski	11	3.82	.36	3 - 5	22	3.34	.34	2.5-5
Remont	20	3.75	1.04	2 - 5	20	3.83	.74	2.5-5

[1] Disease severity scored as follows: 1 = no spread of discoloration; 2 = spread of discoloration of less than 1 cm; 3 = spread of discoloration of more than 1 cm; 4 = extensive vascular necrosis; and 5 = dead plant.

[2] Disease severity scored on spread of discoloration from point of inoculation as follows: 1 = no spread; 2 = 0.1 - 0.9 cm; 2.5 = 1.0 - 1.9 cm; 3 = 2.0 - 2.9 cm; 3.5 = 3.0 - 3.9 cm; and 4 = extensive vascular necrosis; and 5 = dead plant.

CONCLUSIONS

The root-cut-soak inoculation technique is effective for screening sainfoin plants for resistance to the root and crown rot caused by F. solani. Current breeding material, released cultivars, and introduced accessions differ in their disease resistance but none of these sources are immune to the disease. Most rapid progress could be obtained by selecting the best plants from the most disease resistant sources. These selections should be recombined and their progeny screened in repeated cycles of selection. Improved populations should be evaluated under field conditions to insure that selection in the greenhouse is improving longevity in the field. Care should be taken to insure agronomic qualities are maintained during the selection procedure.

Studies should be initiated to characterize the mechanism(s) of disease resistance and its inheritance.

SUMMARY AND CONCLUSIONS

Root and crown rot limits the production of sainfoin. This disease reduces the longevity of the stand and severely reduces forage yield. The objectives of this study were to: 1) determine the causal organism(s) of root and crown rot; 2) further characterize the host-parasite relationships; and 3) develop and initiate screening procedures to detect potential sources of disease resistance.

F. oxysporum infected sainfoin but caused only limited symptom development. Several 'formae speciales' of F. solani infected sainfoin and caused disease symptoms very similar to those observed under natural conditions. This explains the presence of this disease in areas where sainfoin had never been cultivated. Due to the longevity of chlamydospores in the soil, it is unlikely that crop rotations with legumes would effectively control this disease.

Microconidia and macroconidia of F. solani are capable of infecting and causing disease symptoms in sainfoin. The four isolates of F. solani evaluated in this study are equally pathogenic.

The results indicate that F. solani is probably not seed-borne. F. oxysporum and Alternaria spp. were found on the untreated seed. Removing the seed-pod and surface-sterilization eliminated these fungi from most of the seed lots. Alternaria spp. were shown to reduce emergence in autoclaved soil.

Soil fumigation with chloropicrin, soil treatments with the fungicide Benlate, and seed treatment with Benlate failed to reduce disease development.

Development of cultivars of sainfoin which are resistant or tolerant to root and crown rot caused by F. solani offer the greatest potential for control of this disease. The root-cut-soak inoculation technique was used to successfully screen plants in the greenhouse. This technique had good repeatibility and is capable of detecting differences in disease resistance. However, this technique needs to be refined before it is used for screening in the field.

Differences were found in current breeding material, the world collection of Onobrychis, and released cultivars. No source of resistance was immune to the disease, but several sources appeared to have some resistance. Selection and recombination of these plants should result in increased disease resistance.

Future studies should concentrate on 1) defining the mechanism and inheritance of disease resistance; 2) refining selection procedures; and 3) developing highly disease resistant lines of sainfoin.

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