Screening plants for resistance to the bacterial pathogens involved in crown and root rot of sainfoin and alfalfa

by Grace Ann Wegener

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Agronomy
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Abstract:
Sainfoin (Onobrychis viciifolia Scop.) has potential as a valuable forage legume in the western United States. Production, however, is limited because of sainfoin's susceptibility to crown and root rot. The pathogens believed to be involved in this disease are Fusarium solani, F. roseum, Pseudomonas syringae, P. marginalis, and Erwinia amylovora. Recently P. syringae was implicated as the pathogen responsible for the loss of approximately 405 ha of alfalfa. Therefore, greenhouse studies were conducted to determine the pathogenicity of P. syringae to alfalfa.

The objectives of this study were to (1) develop an effective greenhouse screening technique to find plants resistant to P. syringae (sainfoin and alfalfa) and E. amylovora (sainfoin), (2) determine the effect of multi-pathogen inoculation (sainfoin), and (3) determine the value of artificial inoculation in a field situation (sainfoin).

Three inoculation techniques were evaluated. A crown injection technique was the most effective in producing disease symptoms in sainfoin and alfalfa seedlings. It resulted in high percentages of infection, severe disease symptoms, and low seedling mortality attributable to the inoculation technique.

Differences in disease resistance were detected among half-sib sainfoin families and cuttings from alfalfa clones inoculated in the greenhouse using the crown injection technique, but the repeatibility of the technique was low, which was reflected in low heritabil-ities. Selections should be based on progeny tests and not on an individual plant basis.

Artificial inoculation is not necessary in the field since disease severity was not greatly increased by artificial inoculation.

Differences in disease severity were detected among sainfoin populations grown in the field. SK48 had significantly lower disease severity scores than the other entries. This cultivar should be used as a germplasm source for future breeding programs.

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GRACE ANN WEGENER

A thesis submitted in partial fulfillment
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Approved:

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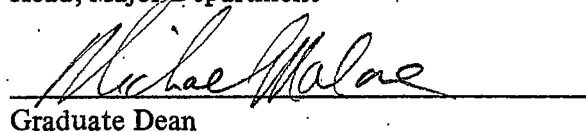
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ABSTRACT

Sainfoin (*Onobrychis viciifolia* Scop.) has potential as a valuable forage legume in the western United States. Production, however, is limited because of sainfoin's susceptibility to crown and root rot. The pathogens believed to be involved in this disease are *Fusarium solani, F. roseum, Pseudomonas syringae, P. marginalis,* and *Erwinia amylovora*. Recently *P. syringae* was implicated as the pathogen responsible for the loss of approximately 405 ha of alfalfa. Therefore, greenhouse studies were conducted to determine the pathogenicity of *P. syringae* to alfalfa.

The objectives of this study were to (1) develop an effective greenhouse screening technique to find plants resistant to *P. syringae* (sainfoin and alfalfa) and *E. amylovora* (sainfoin), (2) determine the effect of multi-pathogen inoculation (sainfoin), and (3) determine the value of artificial inoculation in a field situation (sainfoin).

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Artificial inoculation is not necessary in the field since disease severity was not greatly increased by artificial inoculation.

Differences in disease severity were detected among sainfoin populations grown in the field. SK48 had significantly lower disease severity scores than the other entries. This cultivar should be used as a germplasm source for future breeding programs.

Chapter I

INTRODUCTION

Sainfoin (*Onobrychis viciifolia* Scop.) is a perennial forage legume grown in some areas of the western United States and Canada. It is winter-hardy, drought tolerant, nonbloating, and resistant to the alfalfa weevil (*Hypera postica* Gyllenhal). Sainfoin also has good palatability and nutritional value.

Although sainfoin has many assets, it also has several problems that severely limit its acceptance and production. The most critical problem is stand loss and deterioration three to five years after seeding. Stand reduction and consequent loss of yield appear to be the result of crown and root rot, caused by *Fusarium solani* (Mart.) Appel & Wr., *F. roseum*, *Pseudomonas syringae*, *P. marginalis*, and *Erwinia amylovora*.

If sainfoin is to become an important forage legume, cultivars must be developed that are not susceptible to crown and root rot.

Alfalfa, commonly called 'Queen of the Forages', is one of the most important forage legumes in the world. It is well adapted to many climatic and soil conditions. Alfalfa is highly nutritious, producing more protein per ha than any other forage.

Recently, *P. syringae* was implicated as the pathogen responsible for the loss of approximately 405 ha of alfalfa near Manhattan, Montana.

The objectives of this study were to (1) develop an effective greenhouse screening technique to find plants resistant to *P. syringae* (sainfoin and alfalfa) and *E. amylovora* (sainfoin), (2) determine the effect of multi-pathogen inoculation (sainfoin), and (3) determine the value of artificial inoculation in a field situation (sainfoin).

Chapter II

LITERATURE REVIEW

Description and Agronomic Characteristics of Sainfoin

Sainfoin (*Onobrychis viciifolia* Scop.) is a deep-rooted, perennial forage legume. It has a tap root system extending 1-10 meters deep [65], with nodulation occurring on the many smaller lateral roots [1,7,50,64,65].

Tall, erect, hollow stems arise from a branched crown. The leaves are vetch-like, having 11-29 leaflets [9,63,65]. Pink flowers are born in a raceme-type influorescence with 5-80 flowers/raceme [11]. The seed is kidney-shaped and about three mm long [8]. Each seed is brown to black in color, and born singly in lenticular rough pods with noticeable veination.

Under irrigation, forage yields of sainfoin are similar to those of alfalfa (*Medicago sativa* L.)[9,34,51]. Sainfoin yields more than alfalfa on the first cutting, but less on following cuttings due to slow recovery after harvest [9]. Decrease in sainfoin yields, three to five years after seeding, is attributed to stand loss and deterioration [9,14,34,51].

Sainfoin forage quality is similar to that of alfalfa. It is lower in crude fiber, crude protein, calcium, and ash content, but is higher in nitrogen free extract, total digestible nutrients, and phosphorus [18,42,53].

Seed yields range from 800-1000 kg h^{-1} in Nevada and Idaho [41,51], to as high as 1350 kg h^{-1} in Montana [11].

It is resistant to the alfalfa weevil (*Hypera postica* Gyllenhal), and may be grown when the weevil limits alfalfa production [8,20]. In addition, sainfoin is nonbloating,

winter-hardy [8,13,22], and grows well in the dry calcareous soils of the northern Rocky Mountain region [8,20,34,67].

History and Distribution of Sainfoin

Sainfoin is native to south central Asia and was grown in Russia over 1000 years ago [56,63]. It was cultivated in Europe in the 14th century [1], and was brought to the United States in the late 19th century [21,56].

The cultivar 'Eski' was released by the Montana Agricultural Experiment Station in 1964 [22]. Selections, based on winter-hardiness, were made from a Turkish introduction brought to the United States in 1952 [21].

'Melrose' was the first sainfoin cultivar to be licensed in Canada [12,33]. It is more winter-hardy, taller, flowers earlier, and recovers more quickly after harvest than Eski.

'Remont' sainfoin, a 16 line synthetic, was released by the Montana Agricultural Experiment Station in 1971 [10]. Selections made from *Onobrychis* plant introductions were based on winter-hardiness and rapid regrowth after harvest. Remont begins growth earlier in the spring than Eski and flowers the year of seeding.

'Renumex' was released by the New Mexico Agricultural Experiment Station in 1977 [49]. It was developed from a germplasm composite of Eski and Remont. Renumex is similar to Remont, but better adapted to New Mexico's hot growing conditions.

Diseases of Sainfoin

Stem and Leaf Diseases

All reported foliar diseases of sainfoin are caused by fungi. Leaf and stem spot (caused by *Aschochyta onobrychidis*) has been found in England [36], Czechoslovakia [44], and Montana [48]. It is seed-born and survives on crop residue. In Montana, symptoms occur primarily as black lesions on the stem [48].

Leaf spot (caused by *Ramularia onobrychidis* Allescher) has been found in Europe [37]. As the spots enlarge, they become lighter colored in the center. In older spots, sclerotial bodies may form a well defined greyish ring [48].

Another leaf spot (caused by *Septoria orobina* Saac.) is of minor importance and has been reported in England [38]. The spots are fawn colored with brown margins. Pycnidia are embedded within the spots [38,48]. Seed infection occurs, and is an important means of dissemination.

Ring spot (caused by *Pleospora herbarum* Pers. ex. Fr. Rab.; imperfect state = *Stemphylium botryosum* Wallr.) has been found in England [36] and Montana [48]. Symptoms are similar to those caused by *Septoria orobina*. Little economic damage occurs in Montana [48].

Soil-Born Diseases

'Damping off' of sainfoin seedlings is caused by *Alternaria* spp., *Rhizoctonia solani* Kuehn, and *Pythium* spp. [61,69].

The root and crown diseases, to which sainfoin is very susceptible, appear to be the single most important factor limiting sainfoin production [61]. Verticillium wilt (caused

by *Verticillium albo-atrum* Reinke and Berth) has been found in Europe [40,61]. It causes wilting of the leaflets along the midrib [40]. The symptoms are most often observed in warm weather, when plants are under moisture stress [48,61].

Root, crown, and stem rot (caused by *Sclerotinia trifoliorum* Erikss) has been found in Montana [48] and Europe [36]. In Montana [48], diseased plants die quickly, but generally only a few plants in a field are infected.

The most serious disease of sainfoin in Montana is a crown and root rot complex. It is found in both irrigated and dryland areas of the state [2,3,4,30,31,61,62]. It is more severe in irrigated than in dryland fields [2,30,31,61,62]. On dryland fields disease severity increases as annual rainfall increases [61].

Plants grown in moist environments have severe crown decay and vascular discoloration [2,3,30,31,61,62]. The discoloration moves down from the crown into the primary and secondary roots.

Apparently the crown is unable to support the many stems that develop from it and splits [2]. This tearing provides easy entry for pathogens. Infection also appears to occur through the hollow stems which remain after harvest [30,68].

Sears [61,62] concluded that *Fusarium solani* (Mart.) Appel and Wr. was the major causal organism involved in crown and root rot of sainfoin. He found *F. solani* in decayed root tissue at nine of ten locations in Montana. However, efforts to consistently isolate this pathogen from diseased tissue failed.

Auld [2,4] later demonstrated the pathogenicity of *F. solani* to sainfoin, and developed a technique to screen for resistant plants. He evaluated four techniques for inoculating sainfoin seedlings with *F. solani*. These included root-cut-soak, crown injection, aerial

spray, and infested toothpick insertion. The root-cut-soak technique applied to six week old seedlings was superior to the other techniques. It was repeatable, resulted in severe disease symptoms, and the highest number of infected seedlings.

The root-cut-soak technique consists of removing the seedlings from the growth medium, trimming the roots 50 mm below the crown, soaking the roots in inoculum for fifteen minutes, and transplanting the seedlings back into the growth medium. Plant roots were evaluated for disease severity approximately 105 days after inoculation, using a 1-5 scoring index (1 = no disease symptoms, 5 = dead plant).

In 1978, Gaudet [30] took plant samples from two, three, and five year old irrigated sainfoin stands near Bozeman, Montana. The roots were split lengthwise and isolations were made. Very few *Fusarium* spp. were isolated from either the two or three year old plants when the bark was removed from the roots. *F. solani* was recovered in appreciable amounts (32% of the plants) from the five year old stand.

Gaudet [30] postulated that *Fusarium* was not the only cause of crown and root rot. Bacterial pathogens and not *Fusarium* were found at the leading edge of decay. They included: *Pseudomonas syringae, P. marginalis, Erwinia amylovora*-like species, and *E. herbicola*. *E. herbicola* was considered nonpathogenic. Thus, the root and crown rot complex of sainfoin appears to be a complex interaction between *Fusarium* and the three bacteria.

Techniques for Selecting Plants Resistant to Crown and Root Diseases in Other Forage Legumes

Phytophthora root rot (PRR) (caused by *Phytophthora megasperma* Drechsler) is a serious disease of alfalfa, occurring in soils that remain excessively wet for ten days or

more [23,25,26,27,47]. Various techniques are used in screening for resistance to PRR. Pratt et al. [57] inoculated the cotyledons of ten day old alfalfa seedlings with zoospore suspensions of *P. megasperma* by placing a .01 mL drop of zoospore suspension on the tip of each cotyledon. Symptoms on susceptible seedlings, observed 24 h after inoculation, included sunken, necrotic patches. Resistant seedlings exhibited reddish-brown flecking, while the immune plants showed no symptoms. There was a high correlation (r=0.80) between the severity of the seedling reaction and the severity of root rot in eight alfalfa lines and cultivars.

Bray and Irwin [6] inoculated 4-6 week old alfalfa seedlings in flats by pouring inoculum into trenches on each side of the row. The soil was soaked daily to keep it wet. Four weeks after inoculation the roots were rated for disease severity on a 1-5 scoring system (1 = small root lesions, 5 = necrosis of the entire tap root).

Frosheiser and Barnes [27] have made rapid progress in selecting for resistance to *P. megasperma* by seeding alfalfa into a *P. megasperma* infested field disease nursery. Plants are scored on a 1-6 scoring system (1 = no symptoms, 6 = dead plant). Plants selected from the field evaluations are re-inoculated in the greenhouse (to insure that they are not escapes) by placing infested agar on a small taproot wound. The soil is kept near saturation for several weeks, resulting in the death of most of the susceptible plants. Surviving plants are scored similar to the field study. Other workers have also obtained successful results using variations of this technique [17,32,35,39].

Fusarium spp. cause crown and root rots and wilts of many forage legumes [45,46]. Although breeding for resistance to fusarial root rot is difficult due to the many species

and environmental factors involved, several researchers [55,58,68] believe this is a worthwhile undertaking.

Richard et al. [58] evaluated two techniques for screening alfalfa for *Fusarium* root rot resistance. He modified the 'application technique' developed by Leath and Kendall [45]. Richard et al.'s [58] technique consists of placing a polyester strip, laden with inoculum, against the end of the taproot previously trimmed to 30 mm. The plants were then transplanted back into pots.

The second technique, the bare-root-soak, includes removing three week old seedlings from flats, trimming the roots to 30 mm below the crown and soaking them for 2 min in a mycelium and spore suspension. The plants are then placed into plastic pouches containing nutrient solution. One month after inoculation the roots are visually scored for disease severity. Plants that have less than 5 mm of internal discoloration were saved. Both techniques were effective in allowing severe disease symptoms to develop and a high percentage of infection.

Frosheiser and Barnes [28] inoculated ten week old alfalfa seedlings by dipping the bare roots in inoculum for 20-30 minutes. After inoculation, top growth was trimmed to 40 mm and the roots trimmed to 120 mm. The plants were then transplanted in the field. The plants were later scored on a 0-5 scoring system (0 = no discoloration, 5 = stele completely discolored or dead plant). The method was effective in inoculating plants with *Fusarium* and the bacterial wilt pathogen (*Corynebacterium insidiosum* Mcgull, H. L. Jens). Other screening methods used in finding plants resistant to *C. insidiosum* are similar to those used in screening for *Fusarium* resistance [15,24,29,43,54].

Chapter III

EVALUATION OF THREE INOCULATION TECHNIQUES ON REMONT SAINFOIN AND LADAK 65 ALFALFA SEEDLINGS TWO AND SIX WEEKS OF AGE

A useful inoculation technique must be effective in detecting sources of resistance to crown and root rot. The objective of this study was to determine the most suitable seedling age and inoculation technique for screening plants for resistance to *P. syringae* and *E. amylovora* in a greenhouse situation.

Materials and Methods

Dehulled Remont sainfoin seed and Ladak 65 alfalfa seed were surface sterilized in a 0.5% sodium hypochlorite (NaOCl) solution for three minutes. Milk cartons (2.1 liter) were sterilized by washing in a 0.75 NaOCl solution. After air drying, the cartons were filled with sterilized masonry sand and planted with ten seeds of either sainfoin or alfalfa. Seedlings were thinned to five per carton after emergence. The plants were grown in a growth chamber with diurnal temperatures of 18°C (night) and 24°C (day) and a 16 h photoperiod. Lighting was supplied by incandescent and fluorescent bulbs. The seedlings were watered with a nutrient solution (Appendix Table 1) twice daily.

Planting dates were staggered such that two and six week old plants were inoculated on the same day. The following inoculation treatments were used:

Root-Cut-Soak (RCS): Seedlings were removed from the cartons and the roots severed 25 mm below the crown. The seedlings were soaked in a bacterial suspension of approximately 10^8 colony forming units/mL (cfu/mL) for 15 minutes. Top growth was then trimmed to 100 mm and the plants transplanted back into the cartons. This technique was applied to the two and six week old sainfoin and the

six week old alfalfa. Two week old alfalfa plants were too small to inoculate using this technique.

Crown Injection (CI): Two drops of bacterial suspension (10^8 cfu/mL) were injected into the crown of each plant, just below the cotyledons, with a B-D, C-13, 10 cc disposable syringe (22 g 1½ needle). Top growth was then trimmed to 100 mm height. This technique was applied to the six week old sainfoin and alfalfa. The two week old seedlings were too small to inoculate using this method.

Soil Inoculation (SI): One hundred mL of bacterial suspension (10^8 cfu/mL) were poured on and watered into the sand of each carton with 75 mL of tap water. Top growth was trimmed to 100 mm height. This technique was applied to the two and six week old sainfoin and alfalfa.

The above inoculation techniques were also performed on two and six week old check plants using distilled water.

Three experiments using the above inoculation procedures were evaluated using isolates obtained from necrotic crown tissue of sainfoin in 1978.

Experiment 1. Sainfoin seedlings were inoculated with *Erwinia amylovora* isolate #15-3.

Experiment 2. Sainfoin seedlings were inoculated with *Pseudomonas syringae* isolate #1.

Experiment 3. Alfalfa seedlings were inoculated with *Pseudomonas syringae* isolate #1.

Each experiment was set up as a split-split plot design with four replications (one carton/treatment/replication). Main plots were inoculated vs. noninoculated, sub-plots

were age of seedlings (two week vs. six week old plants), and sub-subplots were the three methods of inoculation. The data were analyzed in two ways. First, as a split-split plot design with inoculated vs. noninoculated as main plots and seedling age as subplots. The sub-subplots included only two methods of inoculation; RCS and SI.

To compare the three methods of inoculation, data from plants inoculated at six weeks of age were analyzed as a 2 X 3 factorial. Factor A was designated as inoculated vs. noninoculated and factor B as the three methods of inoculation.

Data on the following variables (except mortality) were collected eight weeks after inoculation:

Mortality: Number of dead plants per carton determined 10 days after inoculation.

Discoloration: The taproot of each plant was split longitudinally and the length of the discoloration measured from the point of inoculation and averaged for the five plants per plot.

Root Density: A 1-7 visual root density score (1 = no roots, 7 = abundant tap and secondary root system) was given to the group of five plants per carton.

Top Dry Weight: Plant tops (five plants/carton) were severed at the crown, dried for 48 hours at 100°C, and weighed.

Root Weight: Plant roots (five plants/carton) were severed at the crown, dried for 48 hours at 100°C, and weighed.

Results and Discussion

Experiment 1: (Sainfoin inoculated with *E. amylovora*)

Differences were found among the three inoculation methods for mortality, root density, top weights, root weights, and root discoloration measurements (Table 3-1) (Appendix Tables 2 and 3).

No seedling deaths occurred with either the CI or SI techniques (Table 3-1). The RCS technique caused a higher mortality of two and six week old seedlings than the other two inoculation methods (p=0.05). Trimming the roots to 25 mm below the crown resulted in seedling shock and death. Two week old plants inoculated with *Erwinia* using the RCS technique had a higher mortality than those inoculated with distilled water (p=0.01), however, these differences were not found in plants inoculated at six weeks of age. Seedling mortality occurring from the RCS technique is probably due to the physical injury and stress placed on the seedlings.

There were no differences in root density scores for plants inoculated with distilled water or *Erwinia* (Table 3-1). Root density scores for plants inoculated using the RCS technique were lower than for the CI and SI methods (p=0.01). There were no differences in root density scores for the six week old plants using the CI or SI techniques. The lower root density scores for the RCS treatments were expected since the roots were trimmed to 25 mm below the crown.

There were no significant differences for top and root weights between the treatments inoculated with *Erwinia* and those inoculated with distilled water (Table 3-1). Top and root weights were lower with the RCS technique than the CI or SI methods (p=0.01).

Table 3-1. The Effect of Three Inoculation Techniques on the Percentage Seedling Mortality, Root Density Scores, Top Growth Weights, Root Weights and Discoloration Measurements of Sainfoin Seedlings Inoculated in the Greenhouse with *Erwinia amylovora*.

	Seedling Mortality	Root Density Scores	Top Weights (g)	Root Weights (g)	Discoloration Measurement
Treatment					
Inoculation					
E. amylovora	1.06	3.06	0.49	0.34	3.80
distilled H_2O	0.50	3.25	0.56	0.30	1.68
$LSD_{.05}$	1.17	2.23	0.27	0.48	2.30
Age					
2 weeks	0.81	2.69	0.21	0.15	1.90
6 weeks	0.75	3.62	0.79	0.49	3.58
$LSD_{.05}$	0.39	0.72	0.21	0.25	3.79
Method					
Inoculation with *E. amylovora*					
2 weeks:					
Root-Cut-Soak	1.25	3.00	0.10	0.08	5.56
Soil Inoculation	0.00	1.75	0.21	0.13	0.00
6 weeks:					
Root-Cut-Soak	1.00	1.75	0.15	0.08	8.79
Soil Inoculation	0.00	5.75	1.31	1.09	0.85
Inoculation with distilled H_2O					
2 weeks:					
Root-Cut-Soak	0.75	2.50	0.95	0.10	2.06
Soil Inoculation	0.00	3.00	0.44	0.30	0.00
6 weeks:					
Root-Cut-Soak	2.25	2.00	0.25	0.14	4.67
Soil Inoculation	0.00	5.00	1.46	0.68	0.00
$LSD_{.05}$	1.79	1.29	0.37	0.51	1.88
Comparisons of Plants Inoculated at 6 Weeks of Age					
Inoculation with *E. amylovora*					
6 weeks:					
Root-Cut-Soak	1.00	1.75	0.15	0.07	8.79
Soil Inoculation	0.00	5.75	1.31	1.09	0.85
Crown Injection	0.25	5.25	1.51	1.19	13.00
Inoculation with distilled H_2O					
6 weeks:					
Root-Cut-Soak	2.25	2.00	0.25	0.14	4.67
Soil Inoculation	0.00	5.00	1.46	0.68	0.20
Crown Injection	0.25	5.25	1.79	1.47	0.20
$LSD_{.05}$	1.13	1.44	0.49	0.73	3.23

There were no differences between the CI and SI techniques. The lower scores of the RCS treatments were because of root pruning and consequent stunting of the top growth. Top and root weights of two week old plants were significantly lower than those of the six week old plants, due to age differences.

Development of vascular discoloration in plants inoculated at six weeks of age, with *Erwinia*, was greater than in the water-inoculation treatments ($p=0.01$) (Table 3-1, Figure 3-1).

The crown injection technique using *Erwinia* resulted in the greatest amount of root tissue vascular discoloration with very little discoloration in the distilled water check treatments. Good symptoms also developed using the the RCS technique, but approximately 55% of the check plants were also infected. This is probably because stray pathogens can easily enter the wounded root tissue during the healing process.

Only slight symptom development occurred with the SI method, indicating it is ineffective as an inoculation technique. A significant inoculation × method interaction ($p=0.05$) occurred because the pattern of response with the SI method varied from the RCS and CI methods.

Overall, the CI technique appeared to be the most promising because it allowed the highest disease symptom development, lowest mortality, and the lowest contamination in the check treatments.

Experiment 2. (Sainfoin inoculated with *Pseudomonas syringae*)

As in the previous experiment, differences were found among the three inoculation methods for mortality, root density, top weights, root weights, and root discoloration measurements (Table 3-2) (Appendix Tables 4 and 5).

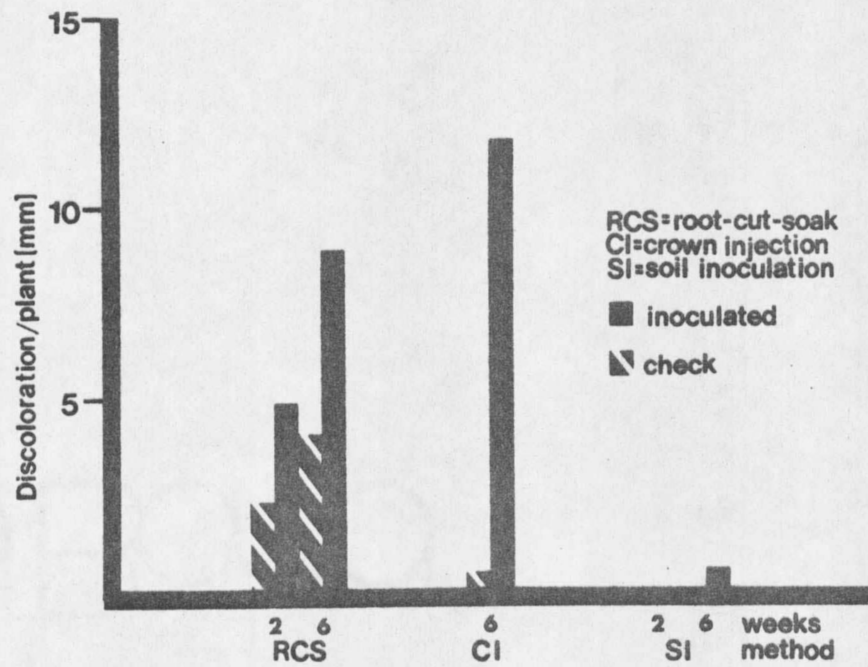

Figure 3-1. The Effect of Three Inoculation Treatments on Internal Root Discoloration of Two and Six Week Old Remont Sainfoin Seedlings Inoculated with *Erwinia amylovora*.

Table 3-2. The Effect of Three Inoculation Techniques on the Percentage Seedling Mortality, Root Density Scores, Top Growth Weights, Root Weights and Discoloration Measurements of Sainfoin Seedlings Inoculated in the Greenhouse with *Pseudomonas syringae*.

	Seedling Mortality	Root Density Scores	Top Weights (g)	Root Weights (g)	Discoloration Measurement
Treatment					
Inoculation					
E. amylovora	0.56	3.94	0.61	0.48	11.56
distilled H_2O	0.75	3.69	0.66	0.38	3.93
$LSD_{.05}$	0.68	2.08	0.44	0.32	10.94
Age					
2 weeks	0.50	3.00	0.32	0.19	2.09
6 weeks	0.81	4.62	0.96	0.69	12.79
$LSD_{.05}$	0.99	5.20	0.28	0.28	7.04
Method					
Inoculation with *E. amylovora*					
2 weeks:					
Root-Cut-Soak	2.50	1.75	0.53	0.08	7.23
Soil Inoculation	0.00	3.50	0.53	0.32	0.00
6 weeks:					
Root-Cut-Soak	1.75	4.00	0.24	0.27	36.24
Soil Inoculation	0.00	6.50	1.63	1.44	2.80
Inoculation with distilled H_2O					
2 weeks:					
Root-Cut-Soak	0.75	2.50	0.12	0.17	1.15
Soil Inoculation	0.00	4.25	0.56	0.30	0.00
6 weeks:					
Root-Cut-Soak	1.25	2.75	0.39	0.20	14.46
Soil Inoculation	0.00	5.25	1.60	0.86	0.10
$LSD_{.05}$	1.61	2.12	0.33	0.36	18.10
Comparisons of Plants Inoculated at 6 Weeks of Age					
Inoculation with *E. amylovora*					
Root-Cut-Soak	2.50	4.00	0.24	0.27	36.24
Soil Inoculation	0.00	6.50	1.63	1.44	2.80
Crown Injection	0.00	5.50	1.35	2.01	18.55
Inoculation with distilled H_2O					
Root-Cut-Soak	1.25	2.75	0.39	0.20	14.46
Soil Inoculation	0.00	5.25	1.60	0.86	0.10
Crown Injection	0.00	5.50	2.02	1.60	8.77
$LSD_{.05}$	1.55	3.15	0.52	1.30	20.36

No seedling deaths occurred with the SI method. The RCS technique caused significantly higher plant mortality than the CI or SI techniques (Table 3-2). There were no differences between the CI or SI techniques. The high mortalities in both the *Pseudomonas* and water-inoculated RCS treatments indicate the deaths were caused by physical injury from the inoculation procedure.

There were no differences in root density scores between the CI or SI treatments (Table 3-2). The root density scores of the plants inoculated at two and six weeks of age, using the RCS technique, were lower than those inoculated using the CI or SI methods ($p=0.10$, $p=0.05$, respectively). These data are similar to the first experiment.

Top and root weights (of the two and six week old seedlings) varied with the inoculation method, resulting in a significant seedling age X inoculation method interaction ($p=0.01$) (Table 3-2).

Top growth weights of the seedlings inoculated at two weeks of age were lower than those inoculated at six weeks of age. Top growth weights of plants inoculated using the RCS technique were lower than either the CI or SI methods ($p=0.01$, $p=0.01$, respectively). There were no differences between the CI or SI treatments.

Root weights of the two week old seedlings were lower than the six week old seedlings ($p=0.01$). The RCS treatments had lower root weights than the CI ($p=0.01$) or SI ($p=0.06$) treatments.

There was less discoloration in the roots of two week old seedlings than six week old seedlings ($p=0.01$) (Table 3-2, Figure 3-2). The RCS and CI methods resulted in more discoloration than the SI technique ($p=0.01$, $p=0.08$, respectively). Inoculation of six

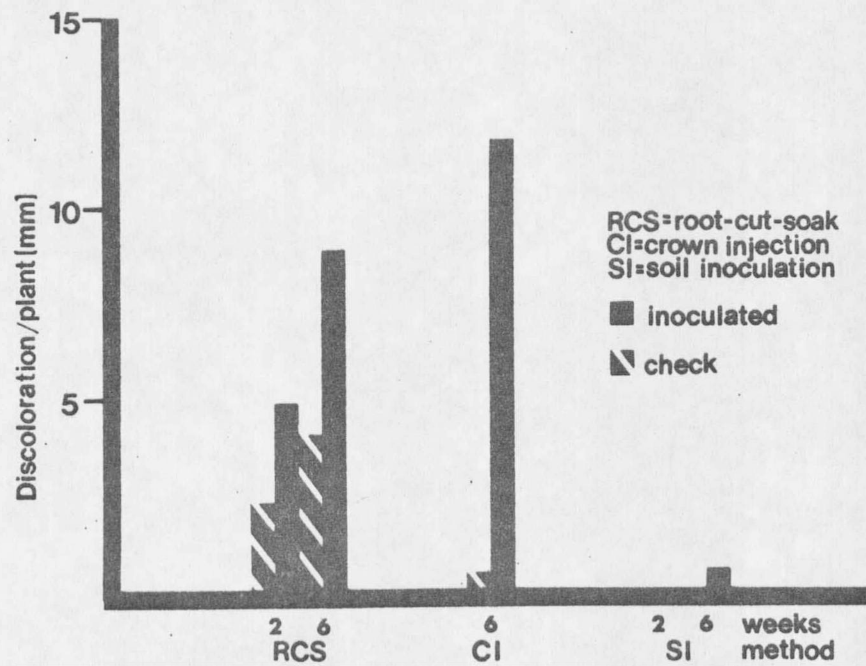

Figure 3-2. The Effect of Three Inoculation Treatments on Internal Root Discoloration of Two and Six Week Old Remont Sainfoin Seedlings Inoculated with *Pseudomonas syringae*.

week old seedlings using the RCS technique caused the highest symptom development to occur. However, contamination of the RCS checks was also high.

The CI method resulted in good symptom development and low contamination of the check treatments. Lack of symptom development with the SI technique resulted in a significant seedling age × inoculation method interaction ($p=0.05$).

Experiment 3. (Alfalfa inoculated with *Pseudomonas syringae*)

Because of the inability to inoculate the two week old alfalfa seedlings using the RCS and CI techniques (the seedlings were too small), only the data obtained from the seedlings inoculated at six weeks of age were analyzed (Table 3-3) (Appendix Table 6).

The mortality rate of the seedlings inoculated using the RCS method were higher than either the CI ($p=0.01$) or the SI ($p=0.01$) methods. There were no differences between the CI or SI techniques. These data are consistent with the two previous experiments.

The root density scores, top, and root weights of the plants inoculated using the RCS method were lower than those inoculated using the CI or SI methods ($p=0.01$). As in the sainfoin experiments, the RCS treatment caused severe injury and stunting.

Discoloration scores were lower with plants inoculated using distilled water than those inoculated with *Pseudomonas syringae* ($p=0.10$) (Figure 3-3). There were no significant differences in discoloration measurements, between the RCS and CI techniques. However, the CI method is preferred because of low contamination of the check treatments and low mortality rates.

Both the CI and RCS methods produced higher disease symptoms than the SI method ($p=0.01$), which is considered ineffective as an inoculation method. These data are similar to the sainfoin experiments.

Table 3-3. The Effect of Three Inoculation Techniques on the Percentage Seedling Mortality, Root Density Scores, Top Growth Weights, Root Weights and Discoloration Measurements of Six Week Old Alfalfa Seedlings Inoculated in the Greenhouse with *Pseudomonas syringae.*

Inoculation Technique	Seedling Mortality	Root Density Scores[1]	Top Weights (g)	Root Weights (g)	Discoloration Measurements (mm)
Inoculation with *P. syringae*[2]					
Root-Cut-Soak	2.75	2.00	0.21	0.27	13.19
Soil Inoculation	0.25	6.00	2.45	2.45	0.00
Crown Injection	0.50	4.50	1.97	2.30	18.39
Inoculation with distilled H_2O[2]					
Root-Cut-Soak	2.25	3.25	0.28	0.38	8.32
Soil Inoculation	0.00	5.25	2.64	2.38	0.00
Crown Injection	0.25	5.75	2.35	2.64	5.38

[1] Scored on a 1-7 scoring system (1 = few roots, 7 = tap and abundant secondary root system).

[2] $LSD_{(p=.05)}$	1.40	2.00	1.46	1.47	9.92

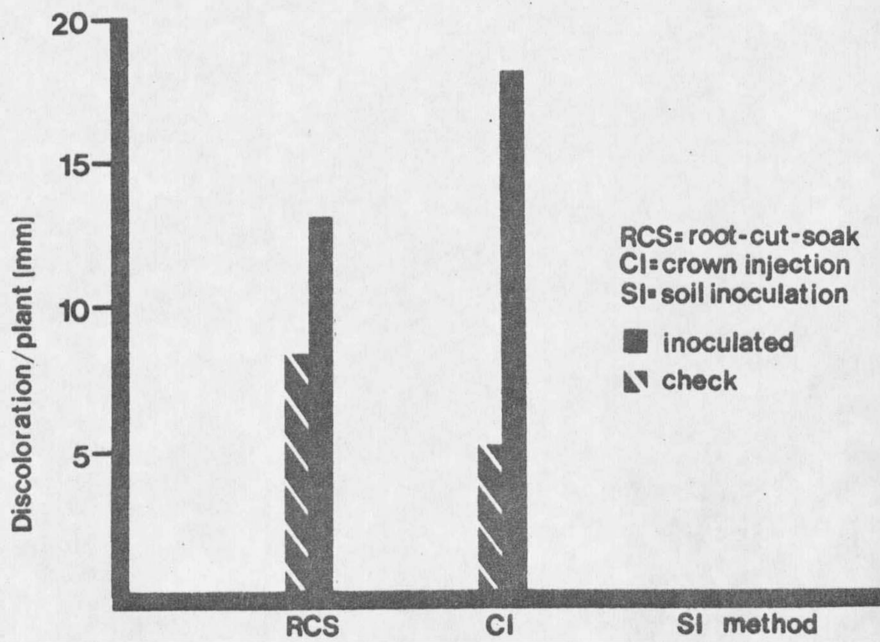

Figure 3-3. The Effect of Three Inoculation Treatments on Internal Root Discoloration of Six Week Old Ladak 65 Alfalfa Seedlings Inoculated with *Pseudomonas syringae*.

Conclusions

The RCS technique causes severe seedling shock and high plant mortality. Contamination was high in the water-inoculated RCS treatments, since there was ample time for stray pathogens to infect the root during the healing process after inoculation.

The SI technique was considered unacceptable for inoculating seedlings due to lack of symptom development.

The crown injection technique appears most appropriate for inoculating seedlings. It resulted in low physical injury to the seedlings, low contamination, and high symptom development in the inoculated treatments.

The six week old seedlings were more suitable for inoculation because (1) the two week old plants were not large enough to inoculate using several of the techniques, and (2) higher disease development occurred with the six week old seedlings.

Inoculating six week old seedlings with *Pseudomonas syringae* or *Erwinia amylovora* using crown injection seems to be the most appropriate procedure and results in severe vascular discoloration.

Chapter IV

THE RESPONSE OF TEN HALF-SIB SAINFOIN FAMILIES AND CUTTINGS FROM NINE ALFALFA CLONES INOCULATED WITH *Pseudomonas syringae* USING THE CROWN INJECTION TECHNIQUE

An effective inoculation technique should produce similar disease severity scores when applied to cuttings of the same clone. The objective of this study was to evaluate the repeatibility of the crown injection technique. Alfalfa clones and sainfoin half-sib families were inoculated with the highly virulent *P. syringae* isolate #1 originally isolated from the crowns of diseased sainfoin plants.

Materials and Methods

In 1981, vegetative cuttings from nine sainfoin and nine alfalfa plants were made. The alfalfa cuttings readily rooted and were transplanted into 100 mm^2 pots containing vermiculite. The sainfoin cuttings did not survive, so ten Remont half-sib families were evaluated instead of clones. Two seeds were planted in each 125 X 25 mm "conetainer" filled with masonry sand. After emergence, the seedlings were thinned to one per conetainer. The plants were grown in the greenhouse at 21°C with a 16 hr photoperiod provided by supplemental lighting from incandescent bulbs. The plants were watered with a nutrient solution once daily (Appendix Table 1).

This study consisted of two separate experiments; nine alfalfa clones and ten half-sib sainfoin families. Each experiment was conducted as a randomized complete block design with four replications. Each replication consisted of either ten half-sib sainfoin families with nine seedlings per entry or nine alfalfa clones with ten cuttings per clone. Data were collected on individual plants within a replication and averaged before being analyzed.

Inoculations were made when the alfalfa and sainfoin plants were six months and six weeks old, respectively. The plants were injected with a 1 mL suspension (10^8 cfu/mL) of *P. syringae* #1 using the crown injection technique described in Chapter III. Top growth was trimmed to approximately 100 mm in height.

Six weeks after inoculation, the plant roots were harvested and evaluated for disease severity. First, the length of internal root discoloration was measured down from the point of inoculation. In addition, a disease severity score was assigned to each root in the following manner: 1 = no discoloration, 2 = faint streak or small spot of discoloration, 3 = heavy line of discoloration, but no lateral spread, 4 = lateral spread of necrosis, 5 = severe necrosis of the entire crown and upper root area (Figure 4-1).

Isolations from diseased root tissue were taken from two plants in each plot and plated on BCBRV agar (Appendix Table 1), a medium selective for fluorescent Pseudomonads.

Results and Discussion

Experiment I: (Sainfoin Half-sib Families)

The crown injection system was effective in producing disease symptoms in the sainfoin half-sib families. *Pseudomonas syringae* was isolated from 98% of the roots.

Differences in disease severity scores were detected among sainfoin half-sib families (Table 4-1). The resolution, however, was limited, with family 52 having a significantly lower score than family 21.

The range of severity scores within each family was high, which is reflected in the low heritabilities. Narrow sense heritabilities were 4.3% and 60.1% when calculated on individual plant and progeny mean basis, respectively (Appendix Table 7).

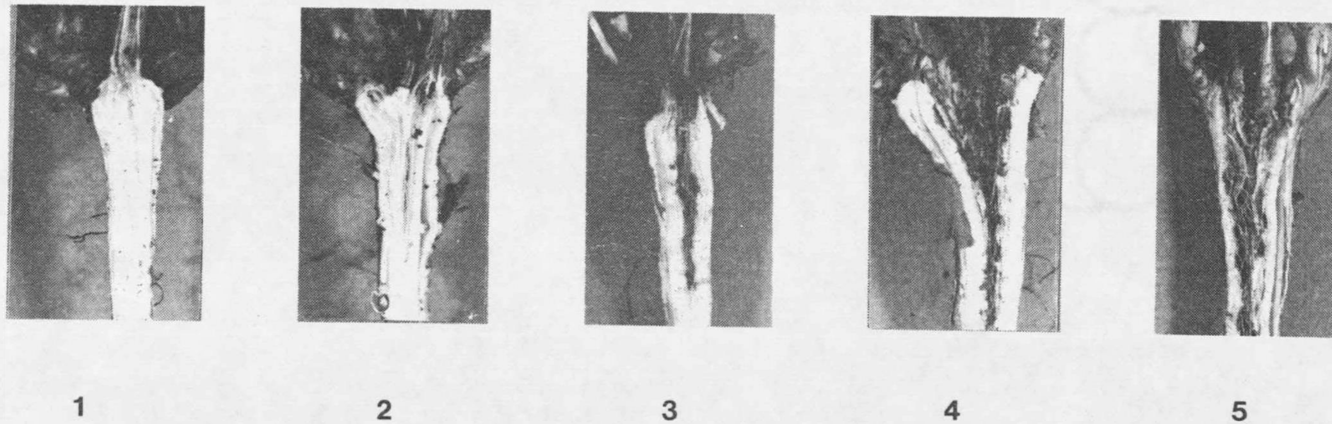

Figure 4-1. Visual Scoring System Used to Assign Disease Severity Scores to Sainfoin and Alfalfa Roots Inoculated with *Pseudomonas syringae* Using the Crown Injection Technique. Numbers refer to the disease severity score assigned to each plant: 1 = no discoloration, 2 = faint streak or small spot of discoloration, 3 = heavy line of discoloration, but no lateral spread, 4 = lateral spread of necrosis, 5 = severe necrosis of the entire crown and upper root area.

Table 4-1. Disease Severity Scores and Length of Root Discoloration of 10 Half-Sib Sainfoin Families Inoculated with *Pseudomonas syringae* Using the Crown Injection Technique.

		Disease Severity Scores[1]			Discoloration Measurement[3]		
Half-Sib	# of Plants Evaluated		Within Family Variance	Range		Within Family Variance	Range
52	33	2.6 a[2]	0.751	1-5	17.4 ab[2]	93.92	1-41
11	33	2.7 ab	0.905	1-5	16.7 ab	112.76	1-55
35	35	2.7 ab	1.386	1-5	14.3 a	111.82	1-41
82	36	2.7 ab	0.892	1-5	14.0 a	97.38	1-30
51	34	2.7 ab	0.746	1-4	19.0 b	96.72	2-45
74	35	2.85 ab	0.891	1-5	18.9 b	98.32	1-42
72	33	2.9 ab	1.059	1-5	17.9 b	101.18	1-46
47	34	3.0 ab	0.696	2-4	19.0 b	73.85	2-39
79	31	3.0 ab	0.733	2-5	14.0 a	56.73	2-30
21	34	3.2 b	1.119	2-5	17.6 ab	86.56	3-42
		CV 10.50%			CV 16.00%		

[1] Disease severity scored as shown in Figure 4-1.
[2] Means not followed by the same letter differ at the .05 probability level (DMR test).
[3] The length of internal root discoloration was measured from the point of inoculation (mm).

The second disease evaluation was a measurement of the distance (mm) internal root discoloration had spread from the point of inoculation. The range of these measurements were extremely variable within each half-sib family. Differences in discoloration measurements were detected among families, but the ranking differed from that of the disease severity scores. Half-sib families 35, 82, and 79 had the least spread of discoloration and had significantly less spread than half-sib families 51, 72, 74, and 47. No correlation (r = 0.15) was found between disease severity scores and the length of discoloration. Data obtained from discoloration measurements could be incorrectly interpreted if internal necrosis had spread laterally instead of longitudinally. Also, measuring the length of discoloration is somewhat dependent on how long the root is, and the ability to longitudinally split the entire root. For these reasons, rating the plants on a disease severity index seems more appropriate than simply measuring the length of discoloration. However, because of the discrepancy in the results of these two evaluations (i.e., disease severity scores and discoloration measurements) further studies should be conducted.

Experiment II: (Alfalfa Clones)

The crown injection method was effective in producing disease symptoms in alfalfa clones, with *Pseudomonas syringae* being recovered from 99% of the roots.

Differences in disease severity scores were found among alfalfa clones (Table 4-2). Clones TR2 and TR3 had significantly lower scores than the other clones. However, there was a wide range of scores within each clone, indicating environmental variance was high.

Discoloration measurements within a clone were also extremely variable. Although differences were detected among clones, the ranking varied from that of the disease severity scores. If a program was implemented to breed alfalfa for resistance to *P. syringae* fur-

Table 4-2. Disease Severity Scores and Length of Root Discoloration of Cuttings From Nine Alfalfa Clones Inoculated With *Pseudomonas syringae* Using the Crown Injection Technique.

	Disease Severity Scores[2]			Discoloration Measurement[4]		
Clone[1]	Means	Within Clone Variance	Range	Means	Within Clone Variance	Range
TR2	3.2^{3}_{a}	0.743	2-4	29.8^{3}_{ab}	139.17	4-50
TR3	3.2_{a}	0.541	2-5	25.1_{bc}	105.89	6-55
G.13	3.6_{b}	0.564	2-5	24.3_{bc}	93.72	4-46
G.11	3.6_{b}	0.292	2-4	27.8_{ab}	82.80	10-42
WS131	3.7_{bc}	0.635	2-5	29.1_{ab}	162.12	7-55
SA10	3.8_{bc}	0.589	2-5	34.0_{a}	131.05	6-55
SA	3.8_{bc}	0.387	3-5	30.0_{ab}	103.47	10-55
G.12	4.0_{c}	0.356	3-5	21.0_{c}	54.10	6-36
MR121	4.0_{c}	0.486	3-5	28.7_{ab}	104.05	6-45
	CV 5.48%			CV 15.39%		

[1] Forty plants evaluated of each clone.
[2] Disease severity scored as shown in Figure 4-1.
[3] Means not followed by the same letter differ at the .05 probability level (DMR test).
[4] The length of internal root discoloration was measured from the point of inoculation (mm).

ther studies should be conducted to determine the reliability of a disease severity scoring system. I feel the same reasoning holds for this experiment as for the sainfoin experiment.

Conclusions

The crown injection technique is effective in producing disease symptoms in sainfoin and alfalfa, although the repeatability of the technique is low.

Genetic variability for resistance to *Pseudomonas syringae* does exist among sainfoin families. However, selections should be based on progeny tests and not on an individual plant basis. Sainfoin half-sib families that have been selected for resistance to *Fusarium solani* should be evaluated for resistance to *Pseudomonas syringae* using the crown injection method.

Genetic variability was detected among alfalfa clones. Selection could be most effective when based upon the mean of several cuttings from a clone.

Chapter V

THE EVALUATION OF MULTI-PATHOGEN INOCULATION OF SAINFOIN IN A FIELD SITUATION

Since the root-crown rot disease complex in sainfoin is associated with five pathogens, it would be advantageous to know if any synergistic or antagonistic interactions exist among them. The objective of this study was to (1) determine if disease severity differs with varying inoculum sources (i.e., different combinations of *F. solani, F. roseum, P. syringae, P. marginalis*, and *E. amylovora*) in a field situation, and (2) to determine the value of artificial inoculation in the field.

Materials and Methods

On May 29, 1981, Remont sainfoin was seeded in a Bozeman silt loam soil at the Arthur H. Post Field Experiment Laboratory west of Bozeman, Montana. The plots were seeded at 22.4 kg ha^{-1} on 305 mm centers in 6.1 m rows. One month after seeding, the plants were thinned to approximately one plant every 152 mm.

The experiment was conducted as a randomized complete block design with four replications. The treatments consisted of inoculating the plants with all possible combinations of the five pathogens involved in crown and root rot of sainfoin (Table 5-1). In addition, a field inoculum was prepared from necrotic crown and root tissue obtained from plants in a sainfoin disease nursery at the Field Experiment Laboratory. To prepare this inoculum, diseased tissue was extracted from the vascular tissue of the root with a scapel, ground in a Waring blender and filtered through cheesecloth.

Table 5-1. Crown and Root Rot Pathogens and Pathogen Combinations Used for Inoculating Remont Sainfoin Seedlings in the Field.

Treatment #	Pathogen(s)[1]	Treatment #	Pathogen(s)	Treatment #	Pathogen(s)
1	1	12	2,5	23	2,3,5
2	2	13	3,4	24	2,4,5
3	3	14	3,5	25	3,4,5
4	4	15	4,5	26	1,2,3,5
5	5	16	1,2,3	27	1,2,3,4
6	1,2	17	1,2,4	28	2,3,4,5
7	1,3	18	1,2,5	29	1,3,4,5
8	1,4	19	1,3,4	30	1,2,4,5
9	1,5	20	1,3,5	31	1,2,3,4,5
10	2,3	21	1,4,5	32	field inoculum
11	2,4	22	2,3,4	33	nonwounded check
				34	wounded check

[1] 1 = *F. solani*
2 = *F. roseum*
3 = *P. syringae*
4 = *P. marginalis*
5 = *E. amylovora*

Every other row in the experiment was a check row. Plants in alternating check rows were inoculated with distilled water. The remaining check rows were left undisturbed to determine the severity of the inoculation technique.

The following isolates were used:

Bacteria[1]

1. *Pseudomonas syringae* #1
2. *Pseudomonas marginalis* #8
3. *Erwinia amylovora* #15-3

Fungi

1. *Fusarium solani* #398[2]
2. *Fusarium roseum* #DG38[3]

The Pseudomonads were grown on BC agar (Appendix Table 1). The *Erwinia* isolate was grown on NBYGA agar (Appendix Table 1). Bacterial densities, determined by using a photoelectric colorimeter (Model 800-3), were approximately 10^8 cfu/mL.

The *Fusarium* isolates were grown as shake cultures in a medium developed for promoting growth of Fusaria (Appendix Table 1). Fungal densities (approximately 22×10^6 conidia/mL) were determined by counting the number of conidia in a hemacytometer.

For the treatments involving more than one pathogen, equal quantities of each isolate were combined. Approximately 160 small absorbent cotton balls were soaked with inoculum for each treatment, placed in a zip-lock bag, stored in an ice chest, and taken to the field, where they were used within several hours.

[1] Obtained from necrotic sainfoin crown tissue, 1978.
[2] Isolated from diseased sainfoin tissue.
[3] Obtained from D. Gaudet's sainfoin *Fusarium* isolates.

Inoculations were made by trimming the top growth to about 150 mm, splitting the crown of each plant (approximately 25 mm deep) with a razor blade, and placing a small inoculum-soaked cotton ball in the wound. All plants in each treatment row (approximately 40) were inoculated. Inoculations were made in August, 1981 (three months after seeding). Initial stand counts were taken at that time.

In May, 1982, stand counts were taken to determine if winter kill (attributable to either the inoculation technique or the pathogens) had occurred.

In a preliminary study, 50 random plants (uninoculated) were dug during May, 1982, in the same plots, but to the south of the field study. Roots were scored on the 1-5 disease severity scoring system described in Chapter IV, and isolations were made using standard procedures [5,59,60,66]. Selective media (Appendix Table 1) used in the isolations included: (1) BCBRVB, a medium selective for fluorescent Pseudomonads, (2) a high sucrose medium selective for *Erwinia amylovora*, and (3) Nash and Snyder's [52] medium, selective for *Fusarium* spp. Two root pieces per plant were used in the fusarial isolations, one with the outer bark (consisting of the phloem, cortex, and periderm) intact and one with the outer bark removed, to determine if *Fusarium* is found in the discolored vascular tissue.

In June, 1982, nine plants per treatment row were dug and rated on the 1-5 disease severity scoring system. Isolations were then made from diseased root tissue of three plants per treatment row (two replications) using the procedure described above.

Results and Discussion

Preliminary Study

All fifty uninoculated plants dug at random exhibited disease symptoms (Table 5-2). Disease severity scores ranged from 2 to 4.

Table 5-2. Disease Severity Scores of Fifty Naturally Infected Remont Sainfoin Plants Grown at the Field Laboratory Near Bozeman, Montana.

Number of Plants	Disease Severity Score
0	1
25	2
15	3
10	4
0	5

Forty-four percent of the plants were infected with *P. marginalis*, 76% with *P. syringae*, and 86% with *E. amylovora*.

When isolations were made with the outer bark of the root intact, *Fusarium solani* was isolated from 36% of the plants and *F. roseum* was isolated from 10% of the plants. When the outer bark was removed, *F. solani* and *F. roseum* were isolated from 0% and 4% of the plants, respectively. These data are consistent with the data reported by Gaudet [30,31], indicating *Fusarium* spp. is primarily confined to the outer bark tissue.

Multi-Pathogen Inoculum Study

There was no significant winter kill in any of the inoculated or check-row treatments. This indicates that the pathogens or inoculation technique did not weaken the plants (the first winter after inoculation) enough to cause severe winter injury.

Significant differences in disease severity did occur among the inoculated treatments (Table 5-3). A consistent pattern, however, was not detected, and paired-t comparisons between the inoculation treatments containing each pathogen did not reveal any significant synergistic or antagonistic interactions among the five pathogens (Table 5-4). There was no difference in disease severity between the nonwounded and wounded check treatments ($p=0.359$). Under field conditions nonwounded plants became infected as quickly as wounded plants.

Inoculation treatments 2 (*F. roseum*), 10 (*F. roseum, P. syringae*), 22 (*F. roseum, P. syringae, P. marginalis*), 23 (*F. roseum, P. syringae, E. amylovora*), 24 (*F. roseum, P. marginalis, E. amylovora*), and 28 (*F. roseum, P. syringae, P. marginalis, E. amylovora*) resulted in greater disease severity than their neighboring check rows (Table 5-5). However, since these treatments were not more severely infected than most of the other treatments (Table 5-3), I hesitate to recommend them as superior inoculums.

Paired-t comparisons of the inoculated treatments and their neighboring wounded and nonwounded check rows did not reveal significant differences concerning the pathogens recovered from the isolations (Table 5-6). Just as many pathogens were recovered from plants in the non-inoculated wounded and nonwounded check rows as the inoculated treatments.

Conclusions

Disease severity and the number of pathogens isolated from diseased root tissue did not increase significantly with artificial inoculation. Apparently, natural inoculum levels are high enough to cause infection in the field.

Table 5-3. Disease Severity Scores of Remont Sainfoin Plants Inoculated With the Crown and Root Rot Pathogens Singularly and in Combination.

Treatment[1] #	Mean[2]	Treatment #	Mean	Treatment #	Mean	Treatment #	Mean
1	2.09	8	2.52	15	2.69	6	2.82
3	2.16	5	2.53	18	2.73	24	2.85
32	2.22	21	2.55	11	2.76	10	2.86
20	2.34	27	2.55	30	2.76	22	2.93
14	2.36	29	2.64	16	2.77	2	2.97
4	2.44	25	2.66	31	2.78	23	3.00
7	2.47	9	2.67	17	2.80	26	3.00
19	2.51	13	2.68	12	2.80	28	3.02

[1] Treatment numbers refer to those shown in Table 5-1.
[2] $LSD_{.05} = 0.46$

Table 5-4. Paired-t Comparisons of Disease Severity Scores Between Inoculation Treatments Containing Each Pathogen.

	Fusarium roseum	*Pseudomonas syringae*	*Pseudomonas marginalis*	*Erwinia amylovora*
Fusarium solani[1]	−0.89	−0.19	0.30	0.36
Fusarium roseum		0.70	0.59	0.53
Pseudomonas syringae			0.11	0.17
Pseudomonas marginalis				0.63

[1] t-Comparisons for each pathogen consisted of all inoculation treatments containing that pathogen (16 treatments/pathogen).

Table 5-5. Paired-t Comparisons of the Disease Severity Scores From Each Inoculated Row With the Two Checks (average score) Neighboring It.

Treatment #[1]	t Value for Comparison	Treatment #[1]	t Value for Comparison
1	-1.836	17	1.103
2	3.990*	18	1.751
3	0.048	19	-0.757
4	-4.296	20	-2.075
5	-0.780	21	-0.344
6	2.519	22	3.771*
7	0.242	23	3.305*
8	1.234	24	4.234*
9	0.754	25	0.448
10	3.605*	26	1.309
11	1.520	27	1.981
12	2.434	28	5.711*
13	1.809	29	2.818
14	-0.361	30	2.326
15	-0.192	31	1.261
16	0.392	32	-1.645

[1] Treatment numbers refer to those shown in Table 5-1.

*Indicates the inoculation was significantly higher than the neighboring checks rows (average score) at the .05 level.

Table 5-6. Paired-t Comparisons of the Bacterial Pathogens Isolated From the Inoculated Rows with the Check Rows Neighboring Them.

Pathogen[1]	t Value for Comparison
Pseudomonas syringae	0.944
Pseudomonas marginalis	1.167
Erwinia amylovora	1.777

[1] t Comparisons for each pathogen consisted of all inoculation treatments containing that pathogen (16 treatments/pathogen).

Wounding the roots did not increase disease severity. The hollow stems appear to be the major infection site, and root wounding did not intensify infection by the pathogens.

Chapter VI

FIELD EVALUATION OF TWENTY-SIX SAINFOIN POPULATIONS FOR RESISTANCE TO CROWN AND ROOT ROT DISEASE

To breed for disease resistance, suitable sources of resistance must be available from which selections can be made. The objective of this study was to determine if levels of resistance to crown and root rot disease varied among sainfoin populations.

Materials and Methods

On May 29, 1981, 26 sainfoin populations (Table 6-1) were seeded in a Bozeman silt loam (Agric Pachic Cryoboral) soil at the Arthur H. Post Field Experiment Laboratory west of Bozeman, Montana. The experiment was seeded at 44.8 kg ha^{-1} in 1.83 m × 6.1 m plots with eleven rows per plot. Two rows of 'Napier' orchardgrass (*Dactylis glomerata* L.) were seeded between individual plots. A randomized complete block design with four replications was used.

In June, 1982, five plants per plot were dug and rated on the 1-5 disease severity scoring system described in Chapter IV.

Results and Discussion

Differences in disease severity were detected among the sainfoin entries (Figure 6-1). There were no significant differences among the Remont types. Eski and EFs1 had significantly higher disease severity scores than ECI and ECIV, but were not different from ECII, ECIII, ECV, and ECVI. Melrose and MFs1 had similar disease severity scores.

After one cycle of greenhouse selection (for resistance to *Fusarium solani*), RFs1 (Remont), EFs1 (Eski), and MFs1 (Melrose) did not exhibit lower disease severity than the

Table 6-1. Sainfoin Populations Evaluated for Resistance to Crown and Root Rot Disease in the Field.

Entry	Background
RCI	Remont – five clones selected for yield
RCII	Remont – five clones selected for yield
RCIII	Remont – ten clones selected for yield
RCIV	Remont – nine clones selected for regrowth
ECI	Eski – five clones selected for yield
ECII	Eski – five clones selected for yield
ECIII	Eski – five clones selected for yield
ECIV	Eski – ten clones selected for yield
ECV	Eski – fifteen clones selected for yield
ECVI	Eski – five clones selected for regrowth
RFs1	Remont – selected for resistance to *F. solani*
EFs1	Eski – selected for resistance to *F. solani*
MFs1	Melrose – selected for resistance to *F. solani*
CC1	Eski – natural selection at Creston, MT
CC2	Eski – natural selection at Creston, MT
BC1	Eski – natural selection at Bozeman, MT
USSR	Russian Bulk (PI no. unknown)
WC	World Collection Bulk
Renumex	Cultivar released by N. Mexico Agr. Exp. Sta.
Eski	Cultivar released by Montana Agr. Exp. Sta.
Remont	Cultivar released by Montana Agr. Exp. Sta.
Melrose	Cultivar released by Canada
W40	Remont-Eski 10 years of natural selection
JB	Judith Basin Bulk
Pola	Polish cultivar
SK 48	Polish cultivar

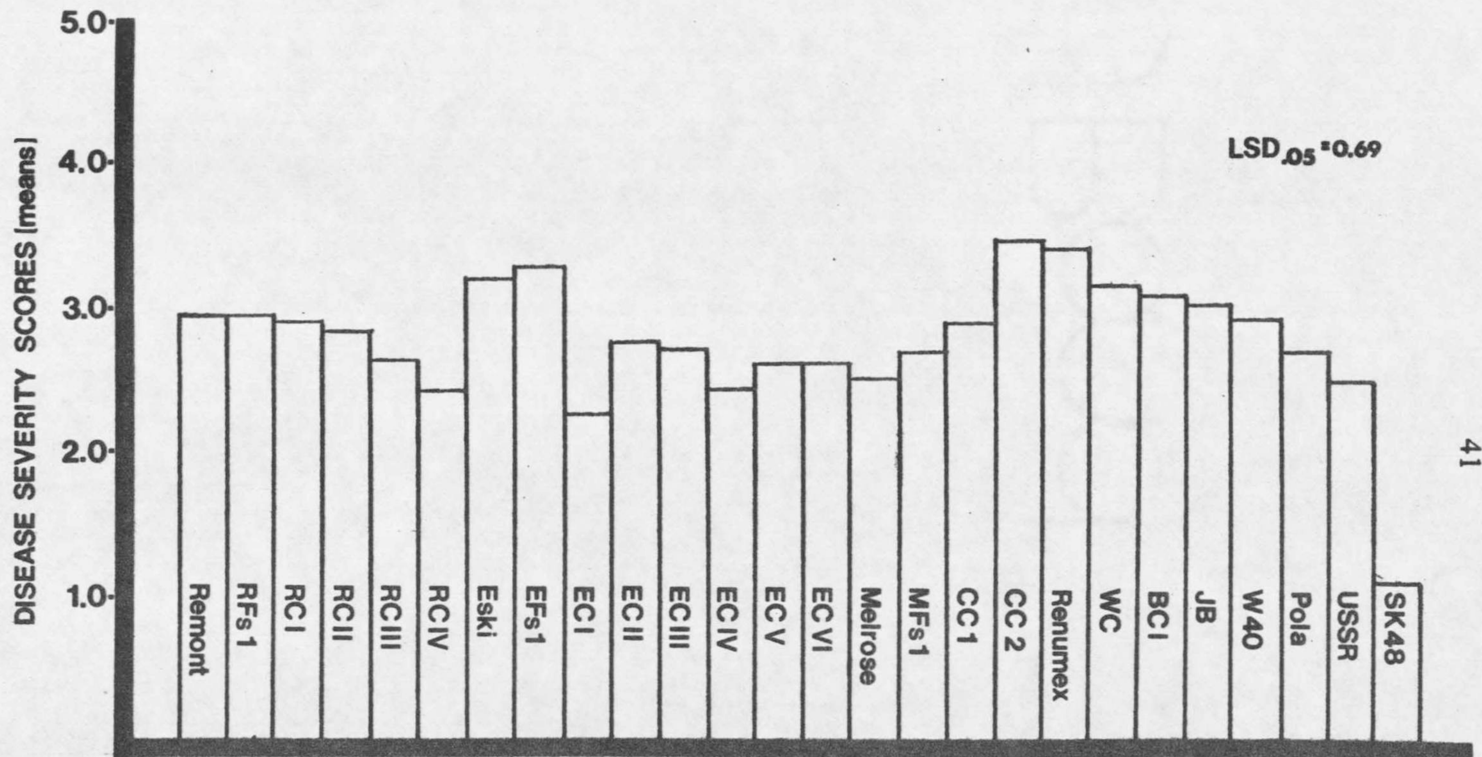

Figure 6-1. The Evaluation of Twenty-Six Sainfoin Entries for Disease Severity When Grown Under Natural Field Conditions (based on 1-5 scoring system, 1 = no discoloration, 5 = severe necrosis of the crown and upper root area).

parental cultivars. This indicates that the bacterial pathogens may be more important in the field than *Fusarium solani*. Selections should also be based on resistance to the bacterial pathogens involved in crown and root rot.

The remaining entries exhibited varying disease severity, with all entries having greater disease development than the Polish cultivar SK48.

Conclusions

Differences in disease resistance existed among the twenty-six sainfoin populations. Progress was not made after one cycle of selection for resistance to *Fusarium solani* in the cultivars Eski, Remont, and Melrose. Greenhouse selections should also include screening plants for resistance to the bacterial pathogens involved in crown and root rot.

Since SK48 had little disease development, selections should be made from this cultivar and tested on a statewide basis to determine its adaptability to Montana's growing conditions.

Chapter VII

SUMMARY AND CONCLUSIONS

Sainfoin (*Onobrychis viciifolia* Scop.) has potential as a valuable forage legume in the western United States. Production, however, is limited because of sainfoin's susceptibility to crown and root rot. The organisms believed to be involved in this disease complex are two fungal (*Fusarium solani* and *F. roseum*) and three bacterial pathogens (*Pseudomonas syringae*, *P. marginalis*, and *Erwinia amylovora*).

Recently, *Pseudomonas syringae* was implicated as the pathogen responsible for the loss of approximately 405 ha of alfalfa near Manhattan, Montana. Consequently, greenhouse studies were performed on alfalfa to determine the pathogenicity of *P. syringae* to this forage species.

The objectives of this study were to (1) develop an effective greenhouse screening technique to find plants resistant to *P. syringae* (sainfoin and alfalfa) and *E. amylovora* (sainfoin), (2) determine the effect of multi-pathogen inoculation (sainfoin), and (3) determine the value of artificial inoculation in a field situation (sainfoin).

The crown injection technique is effective in producing disease symptoms in sainfoin and alfalfa seedlings. It resulted in a high percentage of infection, severe disease symptoms, and low seedling mortality attributable to the inoculation technique. The repeatibility of the technique, however, was low, which was reflected in the low heritabilities. Selections should be based on progeny tests rather than on an individual plant basis.

Artificial inoculation does not appear to be necessary in the field. Disease severity scores of artificially inoculated sainfoin plants were not significantly higher than those in the neighboring check rows, in all but a few instances.

Differences in disease resistance were detected among sainfoin populations growing in the field. SK 48 had significantly lower disease severity scores than all of the other entries. This cultivar would be a good germplasm source for future breeding programs.

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LITERATURE CITED

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APPENDIX

Appendix Table 1. The Composition of the Media Used for Fungal and Bacterial Isolations and Increase, and the Composition of the Nutrient Solution Used in This Study.

I. Fungal Media:	
A. Pentachloronitrobenzene Agar (PCNBA) [52]:	
Agar	20.0 g
Difco peptone	5.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Distilled water	1000.0 mL
Pentachloronitrobenzene (PCNB), 75% W.P.*	1.0 g
Streptomycin*	300.0 mg
Preparation: streptomycin and PCNB are added after autoclaving and medium is cooled to 42-45°C.	
B. *Fusarium* Shake Culture [5]:	
NH_4NO_3	1.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Yeast extract	1.0 g
Distilled water	1000.0 mL
Preparation: Add 15 g carboxylmethylcellulose to the distilled water (slightly warmed) with a stirring bar. Then add 10-100 mg kanamycin sulfate to the culture for bacterial control. Autoclave.	
II. Bacterial Media:	
A. BCBRVB Agar [59]:	
Proteose peptone no. 3	20.0 g
Glycerol	15.0 mL
$MgSO_4 \cdot 7H_2O$	6.0 g
Nobel agar (Oxoid purified agar)	12.0 g
Distilled water	990.0 mL

Preparation: Autoclave at 121°C for 15 min and add growth modifying agents* after cooling to 55-60°C. Agitate and pour plates.

*Growth modifying agents

1. Place 6 mg vancomycin, 10 mg bacitracin, and 0.5 mg rifampicin in 2 mL of 95% ethanol, then add 2 mL of sterile distilled water to the tube.
2. In a separate tube dissolve 500 mg Benlate (50 WP) in 2 mL of 95% ethanol followed with 4 mL of sterile distilled water.

Appendix Table 1 (*continued*)

B. BC Agar	
Proteose peptone no. 3	20.0 g
$K_2HPO_4 \cdot 3H_2O$	2.5 g
$MgSO_4 \cdot 7H_2O$	6.0 g
Cyclohexamide (Sigma)	0.1 g
Nobel agar (Oxoid purified agar)	12.0 g
Glycerol	15.0 g
Distilled water	990.0 mL
C. Nutrient Broth Yeast Glucose Agar (NBYGA):	
Nutrient broth	8.0 g
Glucose	2.0 g
Yeast Extract	5.0 g
Agar	15.0 g
Distilled water	1000.0 mL
D. *Erwinia amylovora* specific medium [16]:	
Sucrose	160.0 g
Nutrient broth	12.0 g
Crystal violet (0.1% in absolute ethanol)	0.8 mL
Cycloheximide (0.1% solution)	20.0 mL
Agar	12.0 g
Distilled water	380.0 mL
III. Nutrient Solution:	
A. Normal Nutrient Solution:	
N	2.3 g
P_2O_5	2.3 g
K_2O	2.3 g
Tap water	18.9 L

Appendix Table 2. Analyses of Variance for Mortality, Root Density, Top Growth Weights, Root Weights, and Discoloration Measurements of Two and Six Week Old Remont Sainfoin Seedlings Inoculated with *Erwinia amylovora* Using the RCS and SI Inoculation Methods.

Source	df	Mortality ms	Root Density ms	Top Growth Weights ms	Root Weights ms	Discoloration Measurements ms
Replications (R)	3	1.08	0.61	0.50	0.12	0.65
Inoculum Source (I) (*Erwinia* or H_2O)	1	2.00	1.53	0.10	0.01	41.54†
E_a	3	1.08	3.95	0.06	0.18	4.18
Seedling age (A)	1	1.13	11.28	2.69**	0.91*	26.83
IA	1	0.13	3.78	0.00	0.14	1.66
E_b	6	0.21	0.70	0.06	0.09	7.19
Inoculation Method (M)	1	18.00**	30.03**	3.99**	1.64**	191.69**
IM	1	0.50	0.03	0.04	0.06	20.58**
AM	1	0.13	19.53**	1.84**	0.85*	10.70**
IAM	1	0.13	2.53	0.16	0.20	0.39
E_c	12	1.35	0.70	0.06	0.11	1.50

†, *, and ** denote significance at the 0.10, 0.05, and 0.01 significance levels, respectively.

Appendix Table 3. Analyses of Variance for Mortality, Root Density, Top Growth Weights, Root Weights, and Discoloration Measurements of Six Week Old Remont Sainfoin Seedlings Inoculated with *Erwinia amylovora* Using the RCS, CI, and SI Inoculation Methods.

Source	df	Mortality ms	Root Density ms	Top Growth Weights ms	Root Weights ms	Discoloration Measurements ms
Replications (R)	3	1.00	0.44	0.70	0.20	0.33
Treatments	5	2.50*	12.87**	1.95**	1.34**	114.13**
Inoculum Source (I) (*Erwinia* or H_2O)	1	0.17	0.17	0.18	0.00	210.46**
Inoculation Method (M)	2	6.00**	31.54**	4.77**	3.09**	103.80**
IM	2	0.17	0.54	0.02	0.25	76.29**
Error	15	0.57	0.91	0.11	0.23	4.59

* and ** denote significance at the 0.05 and 0.01 significance levels, respectively.

Appendix Table 4. Analyses of Variance for Mortality, Root Density, Top Growth Weights, Root Weights, and Discoloration Measurements of Two and Six Week Old Remont Sainfoin Seedlings Inoculated with *Pseudomonas syringae* Using the RCS and SI Inoculation Methods.

Source	df	Mortality ms	Root Density ms	Top Growth Weights ms	Root Weights ms	Discoloration Measurements ms
Replications (R)	3	1.36	2.54	0.34	0.11	137.59
Inoculum Source (I) (*Erwinia* or H_2O)	1	0.28	0.50	0.02	0.03	502.05
E_a	3	0.36	3.42	0.15	0.08	94.53
Seedling age (A)	1	0.78	21.12	3.35**	2.58**	971.85**
IA	1	1.53	8.00	0.00	0.14	149.43
E_b	6	1.32	4.56	0.10	0.11	66.30
Inoculation Method (M)	1	13.78**	36.12**	6.23**	2.83**	1640.93**
IM	1	0.28	0.00	0.02	0.03	345.52
AM	1	0.78	1.13	1.41**	1.66**	732.97**
IAM	1	1.53	0.00	0.01	0.02	70.66
E_c	12	1.09	1.90	0.04	0.05	137.95

** denote significance at the 0.10, 0.05, and 0.01 significance levels, respectively.

Appendix Table 5. Analyses of Variance for Mortality, Root Density, Top Growth Weights, Root Weights, and Discoloration Measurements of Six Week Old Remont Sainfoin Seedlings Inoculated with *Pseudomonas syringae* Using the RCS, CI, and SI Inoculation Methods.

Source	df	Mortality ms	Root Density ms	Top Growth Weights ms	Root Weights ms	Discoloration Measurements ms
Replications (R)	3	0.82	2.28	0.63*	0.04	288.04
Treatments	5	3.07	7.07	2.10**	2.20	1719.71**
Inoculum Source (I) (*Pseudomonas* or H_2O)	1	1.04	4.17	0.41	0.76	782.61†
Inoculation Method (M)	2	6.12*	14.54†	4.78**	4.99**	1142.36*
IM	2	1.04	1.04	0.26	0.13	186.05
Error	15	1.05	4.38	0.12	0.75	182.52

†, *, and ** denote significance at the 0.10, 0.05, and 0.01 significance levels, respectively.

Appendix Table 6. Analyses of Variance for Mortality, Root Density, Top Growth Weights, Root Weights, and Discoloration Measurements of Six Week Old Ladak 65 Alfalfa Seedlings Inoculated with *Pseudomonas syringae* Using the RCS, CI, and SI Inoculation Methods.

Source	df	Mortality ms	Root Density ms	Top Growth Weights ms	Root Weights ms	Discoloration Measurements ms
Replications (R)	3	3.00*	6.60	0.81	1.43	76.42
Treatments	5	5.60**	9.74*	4.95**	4.83**	214.85**
Inoculum Source (I) (*Pseudomonas* or H_2O)	1	0.67	2.04	0.27	0.10	213.19*
Inoculation Method (M)	2	13.62**	20.67**	12.18**	11.95**	344.07**
IM	2	0.04	2.67	0.05	0.08	86.45
Error	15	0.87	2.76	0.94	0.95	43.33

* and ** denote significance at the .05 and .01 significance levels, respectively.

Appendix Table 7. Formulas Used to Calculate the Narrow Sense Heritabilities of Ten Remont Half-Sib Sainfoin Families Inoculated with *Pseudomonas syringae* Using the Crown Injection Technique.

h^2 on an individual plant basis	$\frac{4\sigma^2_F}{\sigma^2_w + \sigma^2_e + \sigma^2_F} = 4.3\%$
h^2 on a progeny mean basis:	$\frac{4\sigma^2_F}{\frac{\sigma^2_w}{rk} + \frac{\sigma^2_e}{r} + 4\sigma^2_F} = 60.1\%$

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