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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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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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).

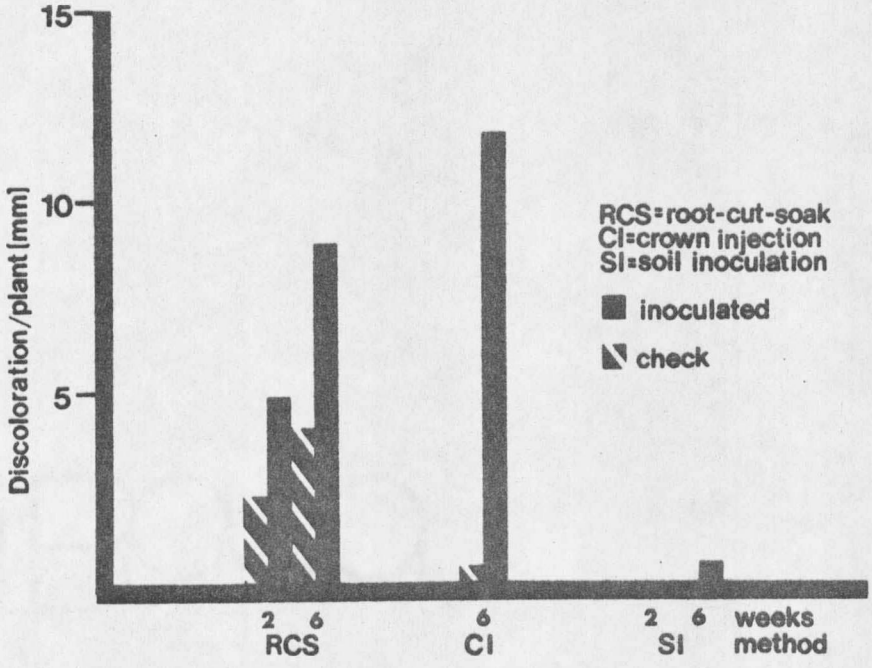

Figure 3-1. The Effect of Three Inoculation Treatments on Internal Root Discoloration of Two and Six Week Old Remont Sainfoin Seedlings Inoculated with *Erwinia amylovora*.