Determining verticillium wilt resistance (Verticillium albo-atrum Reinke & Berth.) in alfalfa (Medicago sativa L.)
by Michael Raymond Bruce

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Agronomy
Montana State University
© Copyright by Michael Raymond Bruce (1987)

Abstract:
Alfalfa (Medicago sativa L.) is the most important forage crop in the world. Most disease and insect problems have been solved through breeding and selection. Verticillium wilt, caused by Verticillium albo-atrum Reinke & Berth. is the most recent disease problem encountered by alfalfa producers. Under cool, moist growing conditions, alfalfa stands may be rendered uneconomical in two to three years. Breeding efforts need to be investigated to prevent yield losses due to Verticillium wilt for irrigated alfalfa produced in the northern United States.

The objectives of this study were to: 1) develop inoculation and scoring age parameters for the root-cut-soak greenhouse selection technique for screening Verticillium wilt resistant plants; 2) investigate the inheritance mode for Vaa resistance; and 3) determine if fungicides can be used to obtain noninfected alfalfa cuttings from infected plants.

Eleven inoculation and ten scoring ages were investigated to determine optimum disease symptom development for plants grown in containers , inoculated with the root-cut-soak greenhouse technique for three cultivars (ML 316, Trumpetor and NC 83-1). Inoculating fourteen week old plants and evaluating them for disease development eight weeks later produced the highest incidence of Verticillium wilt symptoms and lowest percentage of resistant plants in the three cultivars tested.

Progress was observed through one selection cycle for Trumpeter, Ladak 65, NC 83-1, and MTV-1. Population diallel analysis demonstrated the importance of general and specific combining abilities for selecting parents to be used in crosses for Verticillium wilt resistance and indicated potential for improving resistance in adapted cultivars from nonadapted cultivars with higher resistance levels. There appeared to be little maternal or reciprocal influence from the crosses. Inheritance seemed to be primarily conditioned by additive gene action with a slight dominance influence from the diallel analysis, and from crosses made between resistant and susceptible NC 83-1 parents.

Bayleton, Benlate, Tilt and Nuarimol fungicides were investigated for Vaa elimination from infected vegetative alfalfa cuttings. Treatment with Bayleton and Benlate increased cutting survival percentage and Benlate lowered Vaa reisolation percentage, suggesting potential for "cleaning up" alfalfa cuttings with fungicides.

DETERMINING VERTICILLIUM WILT RESISTANCE (*VERTICILLIUM ALBO-ATRUM*

REINKE & BERTH.) IN ALFALFA (*MEDICAGO SATIVA* L.)

by

Michael Raymond Bruce

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Agronomy

MONTANA STATE UNIVERSITY
Bozeman, Montana

May 1987

ii

APPROVAL

of a thesis submitted by

Michael Raymond Bruce

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency; and is ready for submission to the College of Graduate Studies.

May 22, 1987
Date

Raymond L. Littleton
Chairperson, Graduate Committee

Approved for the Major Department

May 26, 1987
Date

Dwane H. Miller
Head, Major Department

Approved for the College of Graduate Studies

_8-17-87_
Date

Wm Malone
Graduate Dean

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the require-
ments for a master's degree at Montana State University, I agree that
the Library shall make it available to borrowers under rules of the
Library. Brief quotations from this thesis are allowable without
special permission, provided that accurate acknowledgment of source is
made.

Permission for extensive quotation from or reproduction of this
thesis may be granted by my major professor, or in his absence, by the
Dean of Libraries when, in the opinion of either, the proposed use of
the material is for scholarly purposes. Any copying or use of the
material in this thesis for financial gain shall not be allowed without
my written permission.

Signature _Michael A Br_

Date _5/22/87_

VITA

Michael Raymond Bruce, son of Mr. and Mrs. William H. Bruce, was born on May 27, 1958 in Missoula, Montana. He received his elementary education at Washougal, Washington and his secondary education at Choteau, Montana, where he graduated from Choteau Public High School in May of 1976. He completed his B.S. in Agriculture - Crop Science Option in June of 1982 from Montana State University. He will complete the requirements for a Master of Science degree in Agronomy in May of 1987 from Montana State University.

ACKNOWLEDGMENTS

TABLE OF CONTENTS

TABLE OF CONTENTS -- continued

LIST OF TABLES

Table   Page

ABSTRACT

Alfalfa (*Medicago sativa* L.) is the most important forage crop in the world. Most disease and insect problems have been solved through breeding and selection. Verticillium wilt, caused by *Verticillium albo-atrum* Reinke & Berth. is the most recent disease problem encountered by alfalfa producers. Under cool, moist growing conditions, alfalfa stands may be rendered uneconomical in two to three years. Breeding efforts need to be investigated to prevent yield losses due to Verticillium wilt for irrigated alfalfa produced in the northern United States.

The objectives of this study were to: 1) develop inoculation and scoring age parameters for the root-cut-soak greenhouse selection technique for screening Verticillium wilt resistant plants; 2) investigate the inheritance mode for Vaa resistance; and 3) determine if fungicides can be used to obtain noninfected alfalfa cuttings from infected plants.

Eleven inoculation and ten scoring ages were investigated to determine optimum disease symptom development for plants grown in cone-tainers, inoculated with the root-cut-soak greenhouse technique for three cultivars (WL 316, Trumpetor and NC 83-1). Inoculating fourteen week old plants and evaluating them for disease development eight weeks later produced the highest incidence of Verticillium wilt symptoms and lowest percentage of resistant plants in the three cultivars tested.

Progress was observed through one selection cycle for Trumpetor, Ladak 65, NC 83-1, and MTV-1. Population diallel analysis demonstrated the importance of general and specific combining abilities for selecting parents to be used in crosses for Verticillium wilt resistance and indicated potential for improving resistance in adapted cultivars from nonadapted cultivars with higher resistance levels. There appeared to be little maternal or reciprocal influence from the crosses. Inheritance seemed to be primarily conditioned by additive gene action with a slight dominance influence from the diallel analysis, and from crosses made between resistant and susceptible NC 83-1 parents.

Bayleton, Benlate, Tilt and Nuarimol fungicides were investigated for Vaa elimination from infected vegetative alfalfa cuttings. Treatment with Bayleton and Benlate increased cutting survival percentage and Benlate lowered Vaa reisolation percentage, suggesting potential for "cleaning up" alfalfa cuttings with fungicides.

Chapter I

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is the most important forage species in the world. Under optimum conditions, it will produce economic yields from four to twenty years [9].

Alfalfa breeding efforts originated in the early 1900's and primarily involved evaluation and mass selection from introduced strains. 'Hardigan', selected for winterhardiness in 1920, was the first cultivar released from selection [73]. In 1940, 'Ranger' was released by the Nebraska Agricultural Experiment Station for resistance to bacterial wilt (*Corynebacterium insidiosum* (McCull.) H. L. Jens.) [73]. Ranger was the first pest resistant cultivar to be released from selection [41,73], and it demonstrated the potential for improving alfalfa through breeding, and since then, many other cultivars have been released in response to disease and insect problems.

Verticillium wilt, caused by *Verticillium albo-atrum* Reinke & Berth. (Vaa), is a recent problem encountered by alfalfa growers in the United States [34]. The disease is devastating in production areas where it is adapted, mostly in the northern United States [21].

Objectives of this study were to: 1) develop inoculation and scoring age parameters for the root-cut-soak greenhouse selection technique for screening Verticillium wilt resistant plants; 2) investigate the inheritance mode for Vaa resistance; and 3) determine if fungicides can be used to obtain uninfected alfalfa cuttings from infected plants.

Chapter II

LITERATURE REVIEW

Verticillium Wilt of Alfalfa

Verticillium wilt of alfalfa was first reported in Sweden in 1918. It was not considered a serious alfalfa disease until its spread continued into northern Europe in the late 1940's and early 1950's [57]. It is now the most important alfalfa disease in Europe [32,57].

Verticillium wilt was first reported in North America in eastern Canada in 1962, but was not detected the following year [6]. The disease was not reported again in North America until 1976, when it was first discovered in the United States in Washington [34]. The next year it was found in southern British Columbia [5]. Since then, it has spread to Wisconsin in 1980 [35]; Idaho, Oregon [21], Wyoming [36], Minnesota, New York, Pennsylvania, and Montana [7] in 1981. In Montana, it was found in Gallatin and Cascade counties in 1981. The following year it was found in Beaverhead, Flathead, Rosebud and Teton counties [27].

This rapid spread causes concern among alfalfa producers and suggests that the fungus is widely adapted. In Washington, the disease was found under environmental conditions ranging from irrigated, alkaline (pH > 7) desert lands to acidic (pH 5-6), high-rainfall coastal areas (over 100 cm annual precipitation). It was not detected on dryland alfalfa fields [21].

Nearly all irrigated alfalfa fields more than one year old in Washington's Columbia Basin contain Vaa infected plants [18,21]. Under extreme weather conditions, complete stand destruction has occurred in two weeks [93]. However, under normal growing conditions, stand life is frequently reduced to uneconomical levels by the second year [32,57] and certainly by the third year [2,5,18,34,57,93].

Verticillium albo-atrum Morphology and Favorable Growth Environments

The causal organisms for various Verticillium wilt diseases have been incorrectly identified, leading to much confusion in the literature. *Verticillium* sp. have similar morphological characteristics and failure to differentiate between them has retarded distinct recognition of geographical and pathological differences among the wilt diseases caused by these fungi [94].

Verticillium albo-atrum belongs to the class Deuteromycetes and no sexual stage has been observed in its reproductive cycle [1]. It is in the order Moniliales, which is characterized by hyaline conidia born on hyphae [1]. *Verticillium albo-atrum* has septate vegetative hyphae which are hyaline or lightly colored [94,99]. It produces verticillately, branched conidiophores in two to three whorls and one to five branches per whorl [21,34,55,75,94,99]. Whorls have enlarged and darkly pigmented basal cells [21,34,85,94,99]. Conidia are born [34,75,94] on straight to slightly curved septate phialides protruding from the conidiophores [94]. Secondary conidia are occasionally produced on phialides that develop from germinating conidia [94]. Conidia are elongate,

hyaline and generally nonseptate [21,34,75,93,98], however, two-celled septate conidia are occasionally produced [85,94].

Verticillium albo-atrum is a nonsclerotial species with a resting mycelium as its survival structure [8,21,34,56,75,94,99]. Resting mycelia originate from a thin-walled, hyaline hyphal mass [94], and then later develop into a darkly colored, thick-walled structure [21,34, 94,99]. Resting mycelium can remain viable in infected seed or soil from nine months to two years [16,21,93].

Verticillium dahliae Kleb. has been often misclassified as Vaa [94]. *Verticillium dahliae* has smaller conidiophores with no basal cell pigmentation. Conidia are smaller and the resting structure is a dark microsclerotium rather than a resting mycelium [94,99]. *Verticillium albo-atrum* grows better under cooler climatic conditions than does *V. dahliae* [69,94].

Best Vaa mycelial growth temperature is between 17-25 C [14,18,19, 25,34,70,80]. Extended exposure to 30 C [56] and 33 C was lethal to the fungus in culture [14,19,70]. Best soil environment for survival and subsequent plant infection is 15-22 C, -30 to -50 megapascals water potential at a pH of 6.5-7.0 [8,61,67]. Under controlled environments, host symptom development is best with 10 hour days and low light intensity (4500 lux) [25,80]. Saturated humidity is required for optimum conidia germination, and fungal growth and development. Thus, the disease is not as severe on dryland fields and seed fields where the plant canopy is less dense [21].

Alfalfa Verticillium Wilt Symptoms

Verticillium wilt diagnosis can be confusing due to similarities of symptoms caused by other pests and nutrient disorders. It is character- ized by individual, seriously affected plants standing among apparently healthy plants [2,21,34,57,85]. Initial symptoms include: temporary flagging and upper leaf wilt on warm days [2,21,34,57,85,93]; yellow, pinkish, or orange-brown leaflet discoloration [2,21,99]; V-shaped nec- rosis from the leaflet tip to its center along the midrib [2,21,85, 89,99]; and leaf curling or cupping upward and inward [21,89,99].

With advanced symptoms, entire leaflets become yellow, then bleach out under rainfall or irrigation, become desiccated and twisted and are easily detached, often with the stems still green and intact [2,21,34, 56,88,92,98]. Under the canopy, more severely affected plants are stunted with most shoots having severe symptoms [2,21,85,89,93,99]; and development of conidia and conidiophores causes a greyish cast to the infected stems [4,34]. The taproot may show yellow to orange vascular discoloration [2,21,34,93]. However, this symptom is not a reliable diagnostic tool, since other diseases can cause the same response and it is difficult to isolate the pathogen from the root [21].

Symptoms may appear late in the planting year, but generally are not apparent until the second year [21]. Best symptom development occurs in early spring, and after each harvest [2,21]. Initial regrowth often appears healthy but the stems quickly die as infection progresses within the plant [21,34,57,59,99]. More stems develop symptoms on

infected plants after subsequent harvests [89]; the plants become prog-

ressively weaker and eventually die over the winter [21,89].

Verticillium Wilt Histology and Disease Mechanism

Verticillium albo-atrum enters the plant through emerging radicals

if the seed is infected or through root and stem wounds in established

plants [48]. The pathogen is disseminated passively throughout the

xylem vessels in the sap stream. Conidia germinate in the vessel ele-

ments and produce mycelia, which in turn, produce more conidia. Conidia

and mycelia lodge at the perforation plate lips causing mechanical water

flow blockage [48,75].

The fungus also induces biochemical changes within the plant after

infection. A heavy molecular weight fraction was discovered through gel

analysis [95] with a constituent of this fraction later determined to

contain cellulase [45,46,64,98]. The cellulase detected was carboxy-

methylcellulase [45]. Cellulase activity breaks down the cell walls,

further restricting vascular flow. Mechanical blockage and cell wall

breakdown leads to plant tissue flaccidity, and ultimately leaf wilting

[47,91]. It appears that the fungus produces cellobiose which facili-

tates cellulase production in the plant [37,45].

Vascular restriction also occurs due to pectinaceous substances.

This response is elicited from the low molecular weight fraction [95],

later determined to contain pectinases and pectin lyases [46,63,64,

65,75,98]. These enzymes attack pectin in the middle lamella of the

vessel element, which causes vascular integrity to be lost [46].

Hydroxyproline [63,65] and other by-products are produced during middle

lamella breakdown, causing a brown, 'gummy' deposit that contributes to vascular plugging [46]. Pectic by-products directly lead to plant tissue necrosis and chlorosis [46].

Although the exact mechanism for resistance is not known, it appears that resistant plants are tolerant to the fungal toxins produced [78]. Protein content is suppressed in susceptible plants [37,98]. Protein content remains near normal in tolerant plants, however fungal growth within the plant is not suppressed and thus some symptoms are evident [37]. Protein content in resistant plants is normal with no fungal presence being detected [37]. The polygalacturonase content (pectic enzyme) is actually higher in resistant plants and activity is increased with available calcium [64,65,67]. The plants may be using a calcareous sidechain to block the polygalacturonase activity, thus leaving the enzyme inactive in the plant solution.

One mechanism that alfalfa uses for resistance against fungi is the production of phytoalexins, which are produced in response to fungal contact. However, during initial infection, pathogenic Vaa strains induce phytoalexin production at a lesser rate than do other fungi [30, 31,62,66]. Later when the plants produce normal phytoalexin levels, there is no apparent reduction in fungal growth. The major phyto-alexins, sativan and medicarpin, also do not deter Vaa fungal growth *in vitro* [30,66]. However, washed conidia were far more sensitive to the phytoalexins than were unwashed [30,31,62]. Thus, it appears that there is a buffer coating that protects Vaa from phytoalexins during its initial growth and the fungus is resistant to alfalfa phytoalexins after they become established within the host.

Verticillium albo-atrum Dissemination

Verticillium albo-atrum is primarily disseminated as conidia and infection occurs through plant tissue wounds [21]. Primary spread is by infected seed and plant debris carried with the seed [2,4,7,15,16,17,21, 50,52,57,58,83,85,93]. Soilborne Vaa can become a primary inoculum source once a field becomes infested [2,4,47,57]. Introduction into the soil occurs by infested plant debris [2,4,21,57,70,85]; contaminated machinery [2,4,21,50,57] or irrigation waterways [50]. The fungus survives in the soil as resting mycelia, from nine months to two years [2,4,47,57]. However, plant residue from susceptible plants or symptomless carriers can extend fungal soil life [51].

Mycelia and conidia transmitted with seed can be either internal [15,16,17,49,50,52,83,85,93] or external [2,4,15,17,21,49,50,57,58, 85,93]. 'Vernal' seed lots produced in Washington contained 0.03% seed which were internally infected with Vaa [93]. Fungal mycelia, growing between differentiated seed coat cells and within the embryo's vascular tissue, was observed with electron microscopy, [16,52]. Internal infection occurred in 4.4% of the seeds after the pedicels were inoculated prior to pollination [16]. *Verticillium albo-atrum* was isolated from seeds harvested from fields with known infections [83].

There was a high correlation between seed size and percent Vaa infection, with smaller seed having a higher infection percentage [15,16]. Seeds smaller than 0.9 mg had the highest infection proportion; however, larger seed classes had sufficient contamination to make seed size screening an uneconomical control [16].

External seed inoculum contributes more to seed dissemination than internal seed inoculum [17,93]. Twenty-five percent of commercial seed lots tested in Washington had Vaa [17] and all inoculum was removed with surface sterilization. After receiving seed lots with 2.2% external infection from Washington, Canada enacted a mandatory seed treatment law, whereby alfalfa seed can not be bought or sold in Canada until the seed lots have been treated with a fungicide [93].

Secondary spread occurs by contaminated machinery [2,4,21,50,57]; irrigation waterways [50]; root contact [2,21,47,50,57]; insects [42,50, 53,54,55,60]; and wind-blown conidia [2,4,21,24,50,57]. Significant airborne conidia levels were detected two feet above infected alfalfa stands and within the stands [24]. Conidia have been found on pea aphids (*Acyrthosiphon pisum* Harris) and the infested aphids could transmit Vaa to healthy plants [42,54]. Leaf-chewing insects: grasshoppers (*Melanoplus sanguinipes* Fab. and *M. bivittatus* Say); alfalfa weevils (*Hypera postica* Gyllenhal); and woolly bear (*Apantesis blakei* Grote) carry conidia on their body parts and viable conidia were observed in their feces [53]. Fungal gnats *Bradysia* spp. transmitted the pathogen to healthy plants [60]. *Verticillium albo-atrum* was detected on leaf pieces used to construct leaf-cutter bee (*Megachile rotundata* Fab.) cells [55]. Verticillium wilt may be transmitted by other alfalfa predators and beneficial insects as well.

Pathogenicity and Virulence of *Verticillium albo-atrum*

There are conflicting reports about Vaa virulence differences in alfalfa. Virulence differences were detected among alfalfa Vaa isolates

in New Zealand [43]. An early comparison between European and North American isolates found the North American isolates to be more virulent [29]. However, a more recent isolate comparison from the two continents showed no differences in virulence [23]. Other reports support evidence that there are no virulence differences for Verticillium wilt of alfalfa [6,10,12,18,19,26]. However, it appears that there are virulence differences for the alfalfa strain isolates on some alternate hosts [18].

There is a large discrepancy in the host range for the alfalfa strain of Vaa. This is, in part, due to fungal species' misclassification. Older literature incorrectly states Vaa is the causal agent for Verticillium wilt of cotton (*Gossypium barbadense* L.) and tomato, (*Lycopersicon esculentum* L.) [56]. However, microsclerotia were identified as the resting structure which is characteristic of *V. dahliae* and not Vaa [56]. Although Vaa can cause symptoms in tomato, it is not considered to be a true tomato pathogen [3].

Even when the correct organism is identified, reports vary on which broadleaf crop and weed species are susceptible to Vaa (Table 30). Some crops have been reported to be in all three classes: resistant, symptomless carriers, and susceptible to Vaa. Differences in virulence and inoculation techniques for various plant species may be responsible for the inconsistencies. An example of crops reported in all three classes is sainfoin (*Onobrychis viciifolia* Scop.). It appears that isolates have exhibited virulence differences to sainfoin. Other species reported falling into both symptomless carrier and resistant classes are potato, red clover, and sweetclover.

To date, all legumes tested have been reported to be susceptible and/or symptomless carriers to alfalfa Vaa, and should not be used in rotations with alfalfa (Table 30). Other broadleaf crop and weed species could maintain the pathogen's presence within alfalfa production areas, if not properly monitored and controlled. Alfalfa plants tolerant to Vaa may also be a symptomless carriers [3,90]. There have been no reports that monocots are affected by alfalfa Vaa and therefore, cereals may be a good crop to use in rotations.

Verticillium Wilt Control Strategies

Verticillium wilt spreads rapidly after establishment in the field. Preventing fungal introduction into the field is the best control measure [2,49,51,93]. Since seeds from infected lots can transmit the disease, care should be taken to use seed from reliable, clean sources [2,6,21,51,93]. Seed fields should not be irrigated after the seeds begin to mature, to limit the pathogen's ability to develop within the seed tissue [54].

Early fungicide treatments and fumigants had limited success in reducing seedborne inoculum [57,58,59]. Seed treatments, such as Thiram 75 WP and Thiram 320, control external inoculum [51,93]. However, standard seed treatments will not eliminate internally seedborne Vaa inoculum [84]. Some seed treatments are also toxic to *Rhizobium meliloti* Dang. [96]. There may be potential for systemic fungicides to prevent plant infection from internal seedborne inoculum [16].

Peaden and Christen [84] showed that dry heat (75 C for 20 hours) may eliminate internal, seed-borne inoculum. However, difficulties in

verifying effectiveness, quality control, and uniformity occurred. No inoculum was detected within plant tissue after a wet heat treatment at 60 C for ten minutes was conducted [74]. However, the dry heat used in the alfalfa pelleting process (90 C) was determined to be insufficient to completely kill the pathogen within plant tissue.

Cultural controls include: harvesting equipment sanitation [2,51, 54,90]; harvesting clean fields before infected ones [27,82]; crop rotations with nonsusceptible species for two to three years to reduce soilborne inoculum [2,4,42,51,82,90]; controlling susceptible hosts, volunteer alfalfa plants, and symptomless carriers [2,4,51,82]; and not irrigating clean fields with the same water used to irrigate infected fields [51].

Cultural control reduces, but does not eliminate, the inoculum source [88]. Once the disease has been introduced into a production area, resistant cultivars are the best way to minimize economic losses [2,4,12,42,51,88,90,97].

Genetic Potential for Developing Resistance to Verticillium Wilt

A genetic basis for plant resistance was discovered during early studies indicating potential for developing resistant cultivars [32,76, 77,79]. Resistance was determined to be predominately under quantita- tive genetic control and conditioned by additive genetic variation [32,77,79]. Due to the polymeric gene action, progress was made through recurrent selection by cumulative dosage effects [79]. However, due to low heritability, homozygosity for resistance was determined to be difficult to reach, and therefore no advantage would be gained by

selfing [32]. Sixty percent of the variation within replications was due to the environment, and general combining ability (GCA) was more important than specific combining ability (SCA) in diallel crosses [32]. Resistance was determined to be general, and most resistant plants exhibited varying degrees of tolerance rather than complete immunity [66].

Viands [97] compared the resistant gene mechanisms between 'Maris Kabul' and 'Vertus' by evaluating progeny from crosses between resistant and susceptible progeny within both cultivars as well as between the two in diallel designs. Some of the progeny from the Maris Kabul x Maris Kabul crosses exhibited unimodal distribution, suggesting additive gene action. However, some progeny exhibited bimodal distribution which suggests a major, dominant gene effect. All progenies from Vertus x Vertus, and Vertus x susceptible Maris Kabul crosses exhibited unimodal distribution, indicating that resistance in Vertus is conditioned by additive gene action. Progeny from Vertus x resistant Maris Kabul crosses exhibited bimodal distribution. Since both cultivars were developed in Europe and both have high resistance levels, it was suggested that the additive genes could be similar in nature and probably came from the same source. However, there was enough evidence to suggest that Verticillium wilt resistance is conditioned by at least two genetic mechanisms.

Genetic potential for resistance to Vaa has been utilized well in Europe. European cultivars have been North America's best resistance source [39,81]. However, most resistant cultivars have Flemish backgrounds and are somewhat susceptible to bacterial wilt [40]. Since Vaa

is mostly a northern growing area problem, winterhardiness needs to be improved in resistant Flemish germplasm sources. Also, bacterial and Verticillium wilt resistant plants should be selected for simultaneously to prevent a new epidemic from an old problem [27].

An alternative to using European cultivars as a resistance source, is to increase resistance levels present in American cultivars. There appears to be low resistance levels in some American cultivars [27,39, 81]. Increasing the gene frequency within these cultivars may allow Verticillium wilt resistance development without losing other desired characteristics associated with northern growing requirements [28].

Breeding and Selection Procedures for Alfalfa Resistance to *Verticillium albo-atrum*

Original selection procedures involved saving plants that survived in infected fields [76]. Most early progeny evaluations were done with field tests in Vaa 'hotbeds'. However, field procedures have been slow, inconsistent and have resulted in a high proportion of escapes [3,22, 76,86].

Presently, most alfalfa Verticillium wilt resistant evaluation tests use some form of root-soak/root-cut-soak greenhouse inoculation. This involves growing plants in the greenhouse under controlled environmental conditions; uprooting the plants and either severing the roots or soaking without wounding in an inoculum source; transplanting the plants back into the greenhouse under controlled conditions; and then evaluating the symptoms after sufficient infection has occurred (Table 31).

Peaden and Gilbert [87] proposed a uniform test in which plants are grown in flats in the greenhouse under 12 hour days, high light intensity (20,000 lux) and 20-22 C temperature for ten to twelve weeks. Plants are removed from the flats and gathered into 50 plant bundles. The roots are trimmed to 6-7 cm below the crown, and the roots soaked in inoculum (8 x 10^6 conidia ml^{-1}) for 10-15 minutes. They are then transplanted back into flats and grown under low light (4500 lux), 10 hour days at a constant temperature (20-22 C). Disease ratings are made four to six weeks after inoculation with a 1-5 scale where: 1) = no symptom; 2) = 1-2 leaflets on one trifoliate with chlorosis; 3) = distinct symptoms on several stems and leaflets; 4) = severe stunting and plant symptoms; and 5) = dead plant. Plants rating 1 and 2 are considered resistant. This greenhouse method gives the best results from late fall through early spring [87].

Some parameters used in the uniform test have been determined by previous experimentation. Best symptom development occurred with low light intensity (4,500 lux) [25, 80]; 10 hour daylength; and temperatures between 20 and 22 C [80]. An inoculum density of approximately 5 x 10^6 conidia ml^{-1} allows the best symptom development [18,27,71].

Inoculation age, scoring age, inoculation method, inoculum concentration, soaking duration, and disease rating scale vary with investigators (Table 31). Daylength and light intensities were not reported for most studies. Temperatures used for all experiments were between 19 and 25 C. However, one study reported better results were obtained when the day temperature was 30 C and the night temperature was 17 C [23].

Most final disease ratings (Table 31) have been taken 2-3 weeks after first symptoms appear (3 to 10 weeks). Two week old plants had higher resistance ratings and there were more escapes [20,23]. McCaslin's [72] 3-4 day inoculation treatment (removing the radical tip), was time consuming and results were inconsistent.

Disease rating schemes are intended to describe alfalfa populations for their overall disease reaction and to determine the resistant plant percentage within each population. The rating system used by Peaden and Gilbert [87] is the most descriptive, and the classes have less interpretative overlap during scoring.

Studies to determine inoculation age and incubation time have not been reported. These parameters should be determined for each greenhouse method used.

Chapter III

INOCULATION AND SCORING AGE DETERMINATION FOR EVALUATING VERTICILLIUM WILT RESISTANCE IN ALFALFA

Technique efficiency for developing alfalfa disease resistant germ-plasms is measured by total time necessary for one selection cycle and genetic progress achieved. Objectives of this study were to determine optimum: 1) plant inoculation age; and 2) scoring age (weeks after inoculation), for selecting alfalfa plants resistant to *Verticillium albo-atrum*.

Methods and Materials

General Methods and Materials.

Unless otherwise specified, the following procedures, when used, were standard for all studies:

Planting and Plant Maintenance. Alfalfa seed was surface steril-ized in a 0.5% sodium hypochlorite (NaOCl) solution for one minute, rinsed in distilled water for five minutes, and air-dried for thirty minutes. Two seeds were planted 10 mm deep into 25 mm X 160 mm "conetainers"[1] (Ray Leach Co., Camby OR) filled with Sunshine Mix[1]

---

[1] Mention of a trademark, proprietary product, or vendor is included for the benefit of the reader, and does not imply endorsement by Montana State University or the Montana Agricultural Experiment Station to the exclusion of other suitable products.

18

(growth mix). Conetainer racks (200 conetainers rack^{-1}) were embedded
into greenhouse benches containing coarse sandblasting sand, so that the
conetainer bottoms were slightly buried into the sand. This allowed for
root penetration through the conetainer bottoms, into sand, which facil-
itated root severing during inoculation [27]. After emergence, plants
were thinned to one cone^{-1} and watered daily with nutrient solution
(Table 32).

Inoculum Maintenance and Density Increase. Fungal culture main-
tenance and handling was done under sterile laboratory conditions, using
a surface sterilized (0.5% NaOCl), clean air chamber. All laboratory
instruments were sterilized with a gas flame and cooled by flaming off
95% ethyl alcohol (EtOH) [71].

Long-term Vaa cultures were maintained on potato dextrose agar
(PDA) slants (Table 32) up to six months, in a standard refrigerator,
before being transferred (100 mm^2 agar blocks) to new PDA slants
[71]. Ten days prior to plant inoculation, Vaa was transferred (100 mm^2
agar blocks) from slant tubes to 250 ml Erlenmeyer flasks containing 100
ml sterile Czapeks Dox Broth (Table 32). Flasks were then placed on a
rotary shaker for ten days to allow for aeration as the inoculum
increased [71]. Inoculum density after ten days was between 1.0 x 10^8
and 5.0 X 10^9 conidia ml^{-1}, as determined with a hemocytometer.

Seven Vaa isolates were available, but pathogenicity tests showed
no virulence differences between isolates [71]. Isolate 421 was arbi-
trarily selected for use in all studies.

Plant Inoculation and Disease Evaluation. Conidial suspensions were diluted to 5.0 X 10^6 conidia ml^{-1} with water. Conetainer racks, with 200 plants each, were removed from the sand benches, and the roots were severed at the conetainer bottoms (150 mm below the crown). The racks were emersed half-way into a conidial suspension (20 l), in fiberglass tubs (400 x 700 x 500 mm) and the plants soaked for 15 minutes to allow for inoculation. The racks were then embedded back into sand benches, and plant tops were trimmed to a height of 70 mm. Check plants were treated similarly, but tap water was substituted for Vaa inoculum [27]. Plants were irrigated daily with nutrient solution (Table 32).

Plants were assigned disease scores according to the standardized Verticillium wilt test [87]; using a 1-5 scale where: 1) = no symptoms; 2) = 1-2 leaflets on one trifoliate with chlorosis; 3) = distinct symptoms on several stems or leaflets; 4) = severe stunting and plant symptoms; and 5) = dead plants. Only plants in the disease class 1 were considered to be resistant for this study. This is contrary to the standardized test which also includes "2's" as resistant plants.

Fungal Reisolation. Stem pieces (10 mm) were removed with a sterile razor blade from between the crown and first node, after final disease ratings were made. They were surface sterilized in 0.5% NaOCl for 30 seconds, rinsed in sterile water for one minute, blotted dry, and placed on Czapeks agar (Table 32) in sterilized, plastic culture plates (10 x 90 mm). Positive fungal identification was first made visually by observing white, fluffy hyphae, which eventually became dark gray to

black. Identification confirmation was done microscopically by observing slides prepared from positive cultures for the presence of verticillately whorled, hyphal branching and conidia [71].

Trial One.

This study was designed as a randomized complete block with four factors and four replications. Factors included: two inoculation treatments (Vaa and tap water check); three cultivars ('Trumpetor', 'WL 316', and 'NC 83-1'); eight inoculation ages (3, 4, 5, 6, 8, 10, 12, and 14 weeks) and ten scoring ages (5, 6, 7, 8, 9, 10, 11, 12, 14, and 17 weeks after inoculation). The experimental unit was comprised of fifty plants.

Replication one was grown in a controlled environment chamber with low light intensity (approximately 5000 LUX), 10 hour days, and constant 20 C temperature. The other three replications were grown in the green-house, set at 20 C without supplemental lighting. Weekly maximum and minimum temperatures were recorded with a "max/min" thermometer.

Plants were grown in mid-summer to mid-autumn and inoculated in late autumn (with the previously described root-cut-soak technique) to minimize high temperature and long daylength effects on disease progression and symptom development [80]. The 14 week old inoculation age was planted first and decreasing ages were planted subsequently so that all treatments were inoculated at the same time, to reduce environmental variation. Disease scores were assigned after symptoms began to develop using the previously described 1-5 scale.

Reisolation was done after final disease ratings were made. Where possible, approximately 40% of the Vaa inoculated plants within each disease class and 20% of the check plants, including all checks not rated as "1's" (resistant), were randomly selected for fungal reisolation.

Data were analyzed by analyses of variance and mean separations made with Tukey's Studentized Range at the 5% significance level.

Trial Two.

A randomized complete block with four replications and the same four factors (inoculation treatment, cultivar, inoculation age and scoring age) was used. However, all factor levels varied slightly, based on observations made from trial one.

Four inoculation treatments were used: 1) Vaa root-cut-soak; 2) Vaa root-cut-soak plus foliar-stem Vaa inoculum application; 3) tap water root-cut-soak plus foliar-stem Vaa inoculum application; and 4) tap water root-cut-soak (check) only. Foliar-stem inoculation (5.0 x 10^6 conidia ml^{-1}), was done by evenly pouring 2000 ml of inoculum $rack^{-1}$ (approximately 10 ml $plant^{-1}$) over freshly trimmed plant tops.

Cultivars WL 316 and NC 83-1 were used, and inoculation ages 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15 weeks were planted in reverse order, and inoculated on the same date. Plants were scored weekly for disease symptoms from 6 to 13 weeks after inoculation with the 1-5 rating system. Damping off diseases and shading effects caused low emergence and seedling survival for inoculation ages 6-9 weeks. Data from these

treatments were not included in the study. The experimental unit was twenty plants.

Plants were grown from summer to fall so that inoculation occurred in late autumn. All four replications were grown in the greenhouse, and temperature was set at 20 C with no supplemental lighting. Weekly maximum and minimum temperatures were recorded with a max/min thermometer.

Approximately 80% of plants in the disease classes 1-4 were randomly selected for reisolation of the pathogen, after the last scoring age treatment for both Vaa inoculated and check plants. Reisolation from dead plants ("5's") was done the week that they died.

Data were analyzed by analyses of variance and mean separations made using Tukey's Studentized Range at the 5% significance level.

Results and Discussion

Growing plants in conetainers was a restriction imposed on this study by the Montana Agricultural Experiment Station's (MAES) forage legume breeding project. Conetainers allow for: efficient greenhouse bench space use (100 plants ft^{-2}); rapid inoculation time (800 plants $hour^{-1}$ $person^{-1}$); and improved transplant survival into field crossing blocks [27].

Trial One.

After inoculation, weekly maximum and minimum temperatures were monitored and recorded (Table 33). Temperature (between 19 and 21 C) and daylength (10 hr day) remained constant throughout the study, in the