

**DISTRIBUTION OF *VERTICILLIUM ALBO-ATRUM*
ON THE ROOT SYSTEMS OF RESISTANT
AND SUSCEPTIBLE ALFALFA**

by

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~~Distribution of *Verticillium albo-atrum* on the root~~

~~systems of resistant and susceptible alfalfa.~~

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Abstract

The invasiveness of *Verticillium albo-atrum* in roots was compared in clonal populations of phenotypically resistant and susceptible alfalfa plants. Entire root systems from inoculated plants were surface sterilized and plated onto water agar immediately and at weekly intervals for 6 weeks following inoculation. Colonies of *Verticillium albo-atrum* growing from roots were assessed at 3, 6 and 9 days after plating, and were scored as point source or continuous. Colonies were defined based on the length of root from which the colony emanated. Point source colonies were defined as colonies emanating from a length of root less than 1 mm long. Point source colonies may reflect the plant's ability to restrict pathogen invasiveness within the root system. Using the ratio of point source to total colonies as a criterion, the root assessments over the 6 week period clearly distinguished the plants as resistant and susceptible and provided a similar differentiation to that provided by conventional foliar symptoms. The results suggest that mechanisms for resistance to *V. albo-atrum* in these two clonal populations operate in both foliar and root tissues. The root assessment technique is a potentially valuable tool for assessing resistance in alfalfa to *V. albo-atrum* and possibly for assessing resistance in other vascular host-pathogen systems.

Dedication

To My Parents

Acknowledgments

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Chapter 1

Introduction

Importance of Alfalfa in Canada

Alfalfa (*Medicago sativa*), is probably the world's most important forage crop (Graham et al 1979), and its designation as the Queen of Forages (Graham et al 1979) reflects this importance. Native to the region around Iran, alfalfa was probably cultivated before recorded history (Bolton et al 1972, Graham et al 1979). Alfalfa is the most important forage legume in Canada (Heinrichs et al 1972).

Alfalfa is largely a crop of temperate regions of the world and the total cropping area devoted to alfalfa is over 33 million hectares (Bolton et al 1972). In 1990 approximately 5.9 million hectares (Statistics Canada) of tame hay was grown in Canada, an earlier study estimated that 56% of the tame hay grown contains alfalfa in mixed or pure stands (Harvey and Atkinson 1983), using this figure gives an estimate of 3.3 million hectares for alfalfa grown in mixed or pure stands in Canada in 1990.

The direct value of alfalfa results from its lifetime yield of high protein forage. Normally the productive life of alfalfa stands extends for a period of 5 or 6 years. In the year of seeding, alfalfa growers incur a net loss of approximately \$300 to \$370 per hectare because alfalfa establishes slowly and yields little in the first year (Harvey and Atkinson 1983). Using the production area of 3.3 million hectares, and a conservative annual hay yield of 6.25 tonnes per hectare (Harvey and Atkinson 1983) valued at an estimated \$60 per tonne, alfalfa represents a \$1.2 billion annual industry.

Another component of the value of alfalfa in Canadian agriculture is its nitrogen fixing capability which is estimated to add \$110 million worth of nitrogen fertilizer equivalents to the soil annually (Harvey and Atkinson 1983). As well, alfalfa forms the basis for some crop rotation schemes, eg. in Southern Alberta, where it is used to clean up

fields of weeds because of its strong competitive ability, and to improve the soils not only by adding nitrogen, but by adding organic matter as well.

Threat of Verticillium Wilt of Alfalfa

Verticillium wilt, caused by *Verticillium albo-atrum*, is a destructive vascular disease of alfalfa. Long recognized as the most important disease of alfalfa in the northern temperate areas of Europe (Graham et al 1977, Atkinson 1983) verticillium wilt was first reported in the United States from the State of Washington in 1976 (Graham et al 1977), and was subsequently found in Canada in British Columbia in 1977 (Graham et al 1977, Atkinson 1983).

Approximately 60% of the Canadian alfalfa crop is estimated to be at risk from verticillium wilt. This percentage includes alfalfa grown under irrigation and in areas where natural moisture is sufficient to favour disease development and spread (Harvey and Atkinson 1983).

Verticillium wilt weakens alfalfa stands and can spread so rapidly within the field that alfalfa yields can be reduced by up to 50% within three years (Atkinson 1983). Losses are attributed to overall reduction of plant and stand vigour and productivity (Harvey and Atkinson 1983). The productive life of the stands is shortened to three years from the customary six years (Atkinson 1983). Reports of the recent experience in the interior of British Columbia and the American Pacific Northwest have confirmed the reputation of verticillium wilt as a very destructive disease (Atkinson 1983).

Causal Agent and Strain Specificity

Verticillium wilt of alfalfa was first reported in Europe in the early 1900's, and it became widespread throughout Europe over the following 25 years. Consistent reports of the disease in North America did not begin until the mid-1970's (Heale 1985).

In Europe, both *Verticillium albo-atrum* and *Verticillium dahliae* are implicated as causal organisms (Isaac 1957). *V. albo-atrum* is very pathogenic to alfalfa under all soil

conditions, whereas *V. dahliae* is virulent to alfalfa plants in soils rich in superphosphate (Isaac 1957). At least in the case of *V. dahliae*, environment has a strong influence on disease expression in the field. The external symptoms of wilt caused by both strains are identical (Isaac 1957). However, in general *V. dahliae* appears to be a milder pathogen than *V. albo-atrum* (Isaac 1957). Both isolates of *Verticillium* studied by Isaac (1957) appeared to be distinct strains differing in their range of pathogenicity from those *Verticillium* strains of both species affecting hop, potato, Brussels sprouts, antirrhinum and tomato.

Verticillium albo-atrum and *V. dahliae* represent the two major verticillium wilt pathogens (Bruehl 1987). *V. albo-atrum* cultures are typically light coloured at first with the mycelium becoming dark as the culture ages. *V. dahliae* produces abundant microsclerotia on most media, and it is this sclerotial formation of *V. dahliae* along with the dark mycelia of *V. albo-atrum* which are the characteristics commonly used to separate these species (Bruehl 1987).

Further investigation into European isolates of both species confirmed that only isolates from alfalfa were virulent on alfalfa with *V. dahliae* continuing to be only slightly pathogenic (Heale and Isaac 1963). However, both strains from alfalfa could infect other crops, namely broad bean, strawberry, Italian clover and pea, while *V. albo-atrum* could also infect potato and runner beans (Heale and Isaac 1963).

The first report concerning verticillium wilt of alfalfa in North America was in 1961 and concerned the reaction of alfalfa to *V. albo-atrum*. It was found that the North American cultivars were not susceptible to *V. dahliae* while only *V. albo-atrum* caused disease (Army and Grau 1985). In North America the causal organism is generally considered to be *V. albo-atrum* (Graham et al 1977), but the European situation indicates the possibility for *V. dahliae* to be involved to some extent.

Early work in the United States indicated that only one alfalfa strain of *V. albo-atrum* exists and that it has potential to become widespread throughout the United States (Christen and French 1982). Further work demonstrated strong likelihood that

North American and European isolates of *V. albo-atrum* were from a common origin (Christen et al 1983). Work going even further in studying the differences between alfalfa wilting and non wilting strains of *V. albo-atrum* showed that crossing alfalfa wilting strains with tomato wilting strains produced recombinants of which only a portion were able to cause either mild or severe disease in both alfalfa and tomato. This implied that more than one gene influenced virulence for the two hosts (McGeary and Hastie 1982).

More recent work investigating *V. albo-atrum* strain specificity further corroborated that the alfalfa strain is the only strain which causes disease on alfalfa in North America, although it can also infect other crops. Alfalfa strains from several North American and European locations were all vegetatively compatible and it was proposed that the alfalfa strain represents a genetically homogeneous clonal population with a common origin (Christen et al 1983, Correll et al 1988).

The inability of the alfalfa strain of *V. albo-atrum* to cause severe wilt in *Antirrhinum spp.* is surprising since this host is used as an indicator for *Verticillium spp.* in general. Such results further emphasize the uniqueness of the alfalfa strain of the pathogen (Heale 1985).

Spread and Dispersal of the Pathogen

The primary means by which *V. albo-atrum* is spread over long distances and is introduced into disease free areas is thought to be through infested seed lots (Howard 1985, Peadar et al 1985). Seedlots can be infested with infected pieces of plant debris such as pods or pedicels or with surface contaminated alfalfa seeds (Huang et al 1985). *V. albo-atrum* association with alfalfa seed can also be internal, between osteosclerid cells in the outer integument of the seed coat (Huang et al 1985, Howard 1985). Infected hay and alfalfa products are other possible vehicles for long distance spread of verticillium wilt of alfalfa (Howard 1985). It has been demonstrated that *V. albo-atrum* can survive the alfalfa dehydration process that is used to produce pelleted alfalfa used for feed (Howard 1985).

Once introduced into an area, verticillium wilt can be disseminated by a myriad of means. Direct root contact can transmit the pathogen from diseased to healthy plants (Howard 1985). Farm machinery and even worker's boots can spread the pathogen within and between alfalfa fields (Howard 1985).

A number of insects have been shown to carry conidia externally on their bodies (Huang et al 1981, Harper and Huang 1984, Harper et al 1988). As well, *V. albo-atrum* has been shown to remain viable in insect feces and able to infect alfalfa plants near the infested feces (Huang and Harper 1985). Even the leafcutter bee (*Megachile rotundata*), the most important alfalfa pollinator, has a role to play in disseminating *V. albo-atrum* in alfalfa (Huang et al 1986a). The bees can carry conidia on their bodies directly or they can carry pollen grains infected with the pathogen. Leafcutter bees can also help spread the disease by using infected leaves to construct their brood cells (Huang and Richards 1983, Huang et al 1986a).

Verticillium albo-atrum has been demonstrated to survive passage through the digestive tract of sheep and infected alfalfa stems in manure piles were shown to harbour viable *V. albo-atrum* six weeks after being buried in the pile (Huang et al 1986b).

On its own *V. albo-atrum* can persist in the soil in the absence of a suitable host for two or three years (Huang and Hanna 1983). However, it has been shown that a number of weed species and other crop plants (ie. tomato, canola) can serve as symptomless carriers of the alfalfa strain of *V. albo-atrum* (Busch and Smith 1982). Even some alfalfa plants can serve as symptomless carriers of the disease (Pennypacker et al 1985). Once introduced into an area, *V. albo-atrum* can settle into a number of reservoir niches which will harbour and maintain the pathogen in the area.

With all the means of dispersal possible for verticillium wilt, the most important means for infection of plants within the field is thought to be via conidia on the cutter bar of the harvester as it moves through the field (Howard 1985, Jimenez-Diaz and Millar 1988).

Pathogenesis

Verticillium wilt is a vascular disease, and as in any vascular disease, the two minimum requirements for disease development are; 1) that the disease has to enter the vascular system of the host, and 2) that once in the vascular system it has to colonize the vascular system to some minimum intensity (Talboys 1964). It appears that in general where *V. albo-atrum* is concerned, root injury facilitates invasion and systemic colonization, and where the alfalfa strain is concerned, plant injury may be essential for systemic colonization. This situation could be modified by the access of the pathogen to exogenous energy sources to aid colonization (Selman and Buckley 1959), ie. previously colonized plants as a base for infection.

Verticillium dahliae has been shown to colonize the roots of a wide range of plant species including both those immune and those susceptible to systemic infection (Gerik and Huisman 1988). Colonization of the roots by *V. dahliae* also occurs primarily near the root tip although the actual entry site for *V. dahliae* into the vascular system is unknown (Gerik and Huisman 1988). *V. albo-atrum* seen in growth pouch studies behaves similarly. Conidia of *V. albo-atrum* were found to germinate and produce hyphae on the surface of the root which began intercellular penetration of the root cap within 24 hours of inoculation. Within three days after inoculation, the fungus had colonized the apical meristem extensively, growing intercellularly, and had penetrated the xylem initials. Where infection occurred behind the root cap the mycelium quickly invaded the cortex and apparently penetrated the xylem vessels from the side and within eight days the fungus could be observed within the xylem vessels of the root (Elango et al 1986). *V. albo-atrum* on tomato and pea gained entry to the vascular system in undamaged roots apparently at the root tips. The root tips of both these species possess no differentiated endodermal cells. In both species the endodermis appeared effective in reducing the number of hyphae that entered the stele in resistant cultivars and to some extent the endodermis could be a determinative region for root infection (Bishop and Cooper 1983).

It appears that *V. albo-atrum* is able to penetrate the vascular system of alfalfa plants through the roots without requiring a wound, although perhaps at a low frequency. Root damage induced by transplanting or by deliberate scalpel cuts resulted in rapid systemic invasion by the fungus (Selman and Buckley 1959). Watering conidial suspensions of the pathogen into soil containing undamaged roots did not usually result in systemic infection (Selman and Buckley 1959). Root penetration does not explain the rapid spread of the disease in alfalfa fields.

In the field, infection and spread of the disease is thought to occur primarily on the aerial plant parts, and it appears that plant injury caused by cutting may promote or be essential for systemic colonization. Conidia of the alfalfa strain of *V. albo-atrum* have been shown to penetrate uninjured healthy alfalfa leaves and stems, but the fungus remained localized at the penetration site even at high conidial concentrations (Jimenez-Diaz and Millar 1986). Systemic colonization occurred only in plants with leaves visibly injured at inoculation, even though the pathogen remained viable in association with the inoculated leaves (both injured and uninjured) for several weeks (Jimenez-Diaz and Millar 1986). These results conflict with the results of Flood and Milton (1982) who reported penetration of alfalfa leaves either directly through or between the epidermal cell walls, and mycelial growth in the palisade and mesophyll tissues associated with little cell death at six days after inoculation.

Research with insect vectors of the disease suggests that biting and chewing insects caused infection in healthy plants via the wound rather than just by their ability to carry the conidia to a host (Harper and Huang 1984). Other research suggests that airborne *V. albo-atrum* conidia or colony forming units (cfu) may not play an important role in the epidemiology of verticillium wilt of alfalfa in the field (Jimenez-Diaz and Millar 1988).

Once in the xylem *V. albo-atrum* is able to rapidly colonize alfalfa plants. Pennypacker and Leath (1983) observed a maximum rate of stem colonization of 354 mm in 16 days in a wilt susceptible plant, which was much faster than mycelial growth on agar. Mycelial growth of the pathogen in culture is quite slow, 70 mm in 20 days on prune-

lactose yeast agar (Pennypacker and Leath 1983). They concluded that the rate of mycelial growth did not explain the rapid rate of colonization in alfalfa plants, and proposed that conidial movement in the xylem was responsible for spread of the pathogen in host plants.

Stems from plants that were infused with a conidial suspension showed dispersal of *V. albo-atrum* to varying distances and in some stems a discontinuous pattern of colonization was detected during the second and third weeks after inoculation (Pennypacker and Leath 1983). Discontinuous patterns of colonization have also been reported in peduncles, pedicels and leaves (Huang et al 1985, Huang 1989), but have not been previously reported in the root system. Spore translocation within the host would lead to a pattern of discontinuous fungal isolations from plant parts.

Eight days after inoculation, numerous conidia were observed in stem xylem vessel elements. Hyphae were frequently observed in conjunction with conidia, possibly indicating colonization of the xylem vessels by hyphae from germinating conidia (Pennypacker and Leath 1983). At this point, conidia within the vascular system were probably produced within the host rather than from the initial conidial suspension used as inoculum (Pennypacker and Leath 1983).

The anatomy of alfalfa allows the fungus to gain access to all the vascular bundles of the crown which are directly connected to the stems (Pennypacker and Leath 1986). However, histological evidence indicated that *V. albo-atrum* has virtually no ability to move between vascular bundles within stems (Huang et al 1985, Pennypacker and Leath 1986).

Verticillium is one of the two most important genera of soilborne fungi responsible for vascular wilt disease, the other being *Fusarium* (Heale and Gupta 1972, Bruehl 1987). The nature of the causal mechanisms of wilting induced by these pathogens, which produce similar symptoms remains a controversial subject although there is a general agreement that the onset of wilting is associated with an overall water deficit (Heale and Gupta 1972).

The two main theories used to investigate the wilting process are the toxin theory and the enzyme theory. The alfalfa strain of *V. albo-atrum* produces both enzymes and toxins which are involved in the disease process. The toxin theory (Gäumann 1958) is that wilting is primarily due to an effect of non-enzymatic substances produced by the pathogen on the leaf cell protoplasts, disrupting their control of water loss (Heale and Gupta 1972).

Toxins have been implicated in *Verticillium* pathogenesis (Nachmias et al 1985, Nachmias et al 1987, Latunde-Dada and Lucas 1988). Toxic fractions of 200 and 400 daltons molecular weight were identified from culture filtrates of the alfalfa strain of *V. albo-atrum* (Latunde-Dada and Lucas 1988). The toxins inhibited alfalfa growth (germination and radical elongation) and caused yellowing and wilting of excised shoots (Latunde-Dada and Lucas 1988).

In verticillium wilt of potato caused by *V. dahliae*, a low molecular weight polypeptide fraction of a protein-lipopolysaccharide complex was identified which was responsible for inducing interveinal chlorosis followed by necrosis when injected into excised potato leaves (Nachmias et al 1985). These symptoms were similar to those seen in potato fields in response to *V. dahliae* (Nachmias et al 1985). It has also been reported that leaf symptoms in *V. dahliae* - infected cotton plants could be caused by low molecular weight phytotoxins (Nachmias et al 1985). The toxic fractions produced by the alfalfa strain of *V. albo-atrum* may be of similar composition to that identified from the isolates of *V. dahliae*. The toxic effects of the alfalfa strain of *V. albo-atrum* were found to be largely reversible when the young alfalfa seedlings were transferred from the culture filtrate to water (Michail and Carr 1966).

The enzyme theory states that wilting is the result of the activity of enzymes produced by the pathogen on the cellular components of the xylem which in turn act to occlude the xylem vessels and block water flow. The enzyme theory rests on a number of lines of evidence; 1) vascular flow rates in the xylem of infected plants are usually reduced as compared with healthy plants, 2) many of the vessels of diseased plants contain dark

gum-like substances or gels that are highly pectinaceous, 3) pectic enzymes have been detected in wilted plants and resistant plants contain less pectic enzyme than susceptible plants and 4) the pectate containing middle lamella is degraded in infected xylem vessels (Heale and Gupta 1972, Durrands and Cooper 1988a).

Several pectic lyase components were detected in alfalfa infected with *V. albo-atrum* and these components were similar to those produced in culture by the fungus (Heale and Gupta 1972). High concentrations of pectic lyase in infected alfalfa coincided with the onset of wilting suggesting that this enzyme may be involved in the process leading to gel blockage of xylem vessels (Heale and Gupta 1972).

Vascular colonization involves breaching of pit membranes and vessel ends and is apparently achieved by localized degradation of this pectin-rich middle lamella primary wall complex (Durrands and Cooper 1988b). In a study of the ability of three pectinase deficient mutants of *V. albo-atrum* to infect and cause symptoms in tomato plants, Durrands and Cooper (1988b) found that symptoms were either absent, less severe, or appeared later than in plants infected with the wild type. Significant extensive colonization was not achieved by a mutant deficient in a range of extracellular cell wall degrading enzymes. It was postulated that a basal level of pectinase is sufficient for penetration into the vascular system, although cell wall degradation may involve the action of a combination of cell wall degrading enzymes (Durrands and Cooper 1988b). With *V. albo-atrum* on hops, there was a strong correlation between virulence group and mean levels of pectic lyase components released into culture fluids after five days growth on hop tissue medium, however the correlation was not absolute (Carder et al 1987). There is still a need for clear evidence to confirm the importance of pectinases in vascular wilt diseases. Pectinases do not seem to be primarily involved in either invasion or nutrition although evidence strongly suggests a role in symptom induction (Durrands and Cooper 1988b).

Both toxins and enzymes produced by *V. albo-atrum* have been able to induce symptoms and cause physical damage in the alfalfa vascular system. It may be, as shown with other diseases, that these pathogen produced metabolites could also suppress phytoalexin production by the plant (Nachmias et al 1987). It is likely that metabolites produced by *V. albo-atrum* could also suppress the physical defense reactions of the alfalfa plant.

Symptoms

Symptoms of verticillium wilt of alfalfa are usually most easily recognized on the regrowth following cutting. They begin to appear when the regrowth is 15 to 20 cm high, and are distinctive in the early bud stage (Christen and Peaden 1981, Yorston 1983). Disease identification in mature stands can be difficult because infected stunted plants are overgrown and symptoms can be confused with wilt symptoms caused by other factors, such as drought, frost injury, bacterial wilt, herbicide damage and boron deficiency (Yorston 1983). Infected plants are usually distributed throughout the field, with one or more shoots on any given plant showing evidence of the disease (Christen and Peaden 1981).

Field symptoms consist of "V" - shaped light coloured necrotic areas at the tips of the leaflets (Christen and Peaden 1981). Leaflets on severely affected shoots are usually necrotic and twist to form spirals (Christen and Peaden 1981). New shoots on infected plants appear normal at first but as they mature, symptoms develop (Christen and Peaden 1981).

Symptoms as seen under controlled conditions may first appear as a yellow blotchiness on the leaflets of a single stem of a plant (Christen and Peaden 1981). The typical "V" - shaped lesion as described in the field symptoms occurs much less frequently (Christen and Peaden 1981). More often, the chlorotic and eventually necrotic lesion begins as yellow streaks along the leaflet midrib and veins. Young leaflets can curl upward and inward from the leaf tip or along the midrib (Christen and Peaden 1981).

Older leaflets remain open as they wilt and become chlorotic (Christen and Peaden 1981); some young leaflets can also take this more open pattern of wilt. Stems typically remain green and erect until all leaves are dead (Christen and Peaden 1981), but occasionally wilt once the wilting of all the leaflets occurs. Symptoms can occur as early as eight days after inoculation in five week old plants (Christen and Peaden 1981).

The symptoms of verticillium wilt, with the exception of the "V"-shaped lesions on the leaflets which do not often occur under growthroom conditions, are non-specific. This is evident by the number of conditions that can be confused with verticillium wilt symptoms. In making determinations as to whether a plant is infected with *V. albo-atrum*, isolation of the pathogen may be required.

Alfalfa Response and Resistance to Verticillium albo-atrum.

Entrance of a foreign microorganism into the vascular elements of a plant elicits a generalized defence reaction in the vascular tissues (Robb et al 1987). The response includes both chemical, ie. phytoalexins, and anatomical factors whose combined function is to limit the spread of the invading organism (Robb et al 1987).

Effective localization includes formation of tyloses or various gums and gels which occlude vessel lumina and apparently block vertical distribution of the pathogen. Lateral invasion of the living tissues is probably limited by callose deposition (Beckman et al 1982, Robb et al 1987), the formation of apposition layers and the secretion of vascular coatings (Robb et al 1987).

Spore trapping at vessel perforation plates and cell end walls is a prerequisite of plant resistance to most vascular wilt fungi (Newcombe and Robb 1988). To escape from the spore trapping site and continue systemic colonization of the plant, the fungus must produce hyphae which either penetrate the end wall and grow vertically into the vessel above the trapping site, or penetrate full bordered pits and grow into adjacent vessels (Newcombe and Robb 1988). In either case, mechanisms of vascular occlusion (ie. gelation, tyloses and phenolic infusion), then intervene in the resistant interaction to halt

the vertical progress of the pathogen in the newly colonized vessels (Newcombe and Robb 1988).

Secretion of vascular coatings is a mechanism of resistance in *Verticillium* infected alfalfa. Coating material apparently forms barriers that prevent lateral invasion by the pathogen into vessels adjacent to trapping sites (Newcombe and Robb 1988). The coating material has a suberin-like nature which apparently makes it highly resistant to degradation and penetration by the fungus (Newcombe and Robb 1988). Following challenge to *V. albo-atrum*, lignin accumulates earlier in resistant alfalfa callus lines than in susceptible lines (Latunde-Dada et al 1987). As well, phenolic compounds accumulated to a higher level in resistant alfalfa genotypes (Christie et al 1985). A fairly high degree of lateral restriction of the pathogen is probably necessary for alfalfa plants to be highly resistant to *V. albo-atrum*, but an efficient coating response alone is not sufficient to prevent disease development (Newcombe and Robb 1988).

Alfalfa also produces phytoalexins in response to invasion by *V. albo-atrum*. Of the five compounds produced in response to *V. albo-atrum*, the two which were produced in the largest quantities were identified as medicarpin (3-hydroxy-9-methoxypterocarpan) and sativan (7-hydroxy 2'-4' dimethylisoflavone) (Khan and Milton 1979). Phytoalexin production is conditioned upon the host's ability to recognize the pathogen. In excised alfalfa leaves inoculated with a conidial suspension of *V. albo-atrum*, the diffusate obtained from the leaves only contained antifungal compounds in significant quantity if the strain used for inoculation was non-pathogenic (Khan and Milton 1975). If conidia of the pathogenic strains were also present in the inoculum, the ability of the non-pathogenic strain to induce phytoalexin production was markedly reduced (Khan and Milton 1975). Flood and Milton (1982) found that alfalfa produced small amounts of phytoalexins in the first few days after a virulent isolate began to colonize the leaf tissue but that development of the pathogen was not affected. Later, after extensive colonization and tissue damage had occurred, the plant did produce high levels of medicarpin and sativan, but was still not able to stop the progress of the pathogen (Flood and Milton 1982). Conversely, the plant

was able to immediately produce high levels of phytoalexin when challenged with the avirulent strain, the result of which was always to cause the death of the pathogen (Flood and Milton 1982). Earlier work has also suggested that alfalfa pathogenic strains of *V. albo-atrum* were relatively insensitive to the antifungal compounds produced by alfalfa (Khan and Milton 1975). Khan and Milton (1975) also suggested that the differential pathogenicity of these strains is closely related to phytoalexin accumulation in the alfalfa plant.

The response time between recognition of the invader and production of a defence response is an important aspect of resistance. A lag phase between penetration and phytoalexin response occurs in alfalfa infected with a pathogenic strain of *V. albo-atrum* (Khan and Milton 1979). The effective resistant response of alfalfa to *V. albo-atrum* probably includes both physical and chemical responses which are initiated quickly after recognition of the pathogen.

Control of Verticillium wilt of Alfalfa

Currently no chemical or cultural control methods in the field will eliminate *V. albo-atrum* from alfalfa seeds or prevent its spread once it is introduced into areas that favour its development (Peadar et al 1985). The pathogen has been difficult to control because of its widespread distribution in soil once it is established in an area, because of its wide host range, and numerous means of dispersal (Peadar et al 1985).

Seed treatments such as benomyl and captan will not protect alfalfa from the pathogen if the seed is internally infected or if the soil is infested with the pathogen (Peadar et al 1985).

The most practical form of control of verticillium wilt of alfalfa is thought to be through the development of resistant alfalfa cultivars (Peadar et al 1985, Busch et al 1985, Ireland and Leath 1987, Grau et al 1991).

Genetics of Alfalfa Resistance to Verticillium albo-atrum

Resistance to *V. albo-atrum* in alfalfa was examined by Panton in the mid sixties and he concluded that it was a multigenic system with either additive or multiplicative effects (Panton 1965, Pennypacker et al 1988). The additive nature of gene effects was confirmed by Nielsen and Andreason (1975). In 1985 Viands concluded that an additive system was operative in Vertus while a dominant resistance gene was present in Maris Kabul (Pennypacker et al 1988). Maris Kabul differs from other alfalfa cultivars by having *Medicago hemicycla*, a wild resistant alfalfa, as one parent (Heale 1985).

It is generally thought that resistance to verticillium wilt is conditioned by several genes (Hawthorne 1987), i.e. that it is polygenic in nature. The lines of evidence put forth to support this are; 1) improved cultivars have shown durable resistance, 2) transgressive segregation occurs, and 3) there is considerable variation in the resistance of individual plants in a population grown from an individual seed lot (Heale 1985).

Review of Techniques used to Evaluate Resistance

Developing methods to assess resistance is essential before breeding for resistance can occur. Methods to assess resistance can be qualitative, i.e. symptom assessments, or quantitative, i.e. some measure of the plant's ability to resist the pathogen.

A number of rating scales have been used to assess resistance in alfalfa based on symptom expression (Graham et al 1977, Busch et al 1985, Pennypacker et al 1985, Hanna and Huang 1987, Ireland and Leath 1987, Hawthorne 1987, Jimenez-Diaz and Millar 1988, Latunde-Dada and Lucas 1988, Newcombe and Robb 1988, Dixon et al 1989, Pennypacker et al 1990, Grau et al 1991). Using visual symptoms to assess resistance has been employed on infected plants in the field (Dixon et al 1989), but more often young plants in controlled environment facilities have been assessed (Pennypacker and Leath 1986, Newcombe et al 1989, Grau et al 1991). The current state of affairs concerning these rating methods is that although they are all similar, they have not been

standardized and all may eventually prove inadequate to assess resistance because of recent work which shows that some alfalfa plants can serve as symptomless carriers of *V. albo-atrum* (Pennypacker et al 1985), and suffer significant reductions in yield compared to disease free plants (Pennypacker et al 1985, Pennypacker et al 1988). Some other, more quantitative measure of resistance will have to be developed to deal with the problem of symptomless alfalfa carriers of verticillium wilt.

Work carried out under controlled environment conditions requires successful artificial inoculation methods. Soaking wounded roots in a conidial suspension of *V. albo-atrum* followed by transplanting into a disease free potting medium is a common method (Pennypacker and Leath 1983, Busch et al 1985, Grau et al 1991), as is applying conidial suspensions via sprays or droplets to cut stubble of alfalfa plants (Pennypacker and Leath 1986, Pennypacker et al 1988, Pennypacker et al 1990, Grau et al 1991). Cut alfalfa stems have also been used to assay resistance to *V. albo-atrum* by subjecting the cut stems to a conidial suspension (Newcombe et al 1989, Newcombe and Robb 1989b).

It is not always necessary to use pathogen propagules when assessing resistance in alfalfa to *V. albo-atrum*. Cut alfalfa stems have been assayed for resistance by soaking them in cell free *V. albo-atrum* culture filtrates (Ireland and Leath 1987). Alfalfa callus tissue has been assayed for resistance to *V. albo-atrum* by monitoring phytoalexin production (Latunde-Dada et al 1987). Toxin fractions of *V. albo-atrum* have also been used to screen alfalfa callus lines for resistance (Latunde-Dada and Lucas 1988).

A more quantitative approach to assess alfalfa resistance to *V. albo-atrum* has been demonstrated using alfalfa stems, by determining the colonization ratio, defined as the number of secondary colonization sites established per original trapping site in a given unit of time (Newcombe et al 1989, Newcombe and Robb 1989b).

A complicating factor in assessing alfalfa resistance to *V. albo-atrum* is the nature of alfalfa populations used in the studies. Alfalfa is a naturally outcrossing tetraploid and as a result inheritance in alfalfa is complex and parent phenotypes do not come true from seed (Busbice et al 1972, Christie et al 1985). Cultivars comprise a highly heterogeneous

mix of plant genotypes and all cultivars contain proportions of "resistant" and "susceptible" plants (Christie et al 1985). To minimize this variability during experimentation, clonal populations of alfalfa must be used when assessing host-pathogen interaction such as assessing resistance.

Objectives

The objectives of this research were to observe, quantify and compare colonization patterns of *V. albo-atrum* invasiveness on the roots of clonal populations of resistant and susceptible alfalfa phenotypes using a root plating technique, and to determine whether this technique could be useful in assessing resistance in alfalfa to *V. albo-atrum*.

Materials and Methods

Inoculum production

The strain of *V. albo-atrum* used was originally isolated from alfalfa and was provided by Dr. H. Huang, Agriculture Canada Research Station, Lethbridge, Alberta. The isolate (designated 1497) characteristically produced dark resting mycelium when grown on potato dextrose agar (PDA). Cultures were maintained on PDA at 22 °C in the dark and were subcultured every 4 months. For inoculum, the fungus was grown in a 200 ml flask containing approximately 150 cc of moist sterilized wheat seed for 3 weeks in the dark at 24 °C. Sterile distilled water was added, and then the contents of the flask were filtered through four layers of cheesecloth the resulting spore suspension was adjusted to 5×10^6 spores/ml.

Production and selection of clonal populations

Two alfalfa plants each from the cultivars Barrier and Pacer were selected from a population of 30 plants which had been grown from seed in a greenhouse for 8 months. Stem cuttings were taken and rooted in a commercial soilless potting medium (Metromix, Grace Horticultural Supply, Vancouver), and grown in a chamber at 20 °C with a 16 h/day photoperiod supplied by fluorescent and incandescent lights ($240 \mu\text{E}/\text{m}^2$), and watered daily as needed. No chemical fertilizers were applied.

After 6 weeks, 24 plants from each of the clonal populations were uprooted and the roots washed free of potting mix. Moisture was blotted from the roots with paper towels, and individual fresh weights of the plants were recorded. The roots were then trimmed in proportion to the size of the plant to include the lower third of the root system. Twelve trimmed plants from each mother phenotype were inoculated by soaking their roots for 1 h in the spore suspension. Another 12 plants had their roots soaked for 1 h in sterile distilled water, and served as controls. The plants were then replanted into individual

5 cm square pots in fresh Metromix, assigned random number identities and arranged in a completely randomized design except that control plants were kept separated from inoculated plants within the same growth chamber at 20 °C, 16 h day (230 $\mu\text{E}/\text{m}^2$) and 18 °C night.

The plants were rated for foliar disease symptoms at weekly intervals for 6 weeks after inoculation. A rating scale adapted from the scales reported by Graham et al (1977), Ireland and Leath (1987) and Pennypacker et al (1988) was used where 1 = no symptoms, 2 = one or two chlorotic leaflets, 3 = chlorotic leaflets on more than one shoot, 4 = most of leaflets chlorotic, 5 = dead plant. Fresh weights of plants from the two most promising clonal populations (one relatively resistant, B09; and one relatively susceptible, P23) were taken at week 6. Stem sections (one from each main stem/plant), 1 - 1.5 cm in length were surface sterilized for 2 min in 1% NaOCl, rinsed in sterile distilled water and plated onto water agar. Any mycelial growth was examined under a dissecting microscope for verticillate conidiophores as a putative indicator of *V. albo-atrum*. In a second experiment the differential interaction phenotypes of selections B09 and P23 were confirmed by repeating the inoculation experiment using 64 plants (32 plants/treatment) from each of the phenotypes B09 and P23 (experiment 2).

Assessment of root invasiveness

A root plating technique (Lévesque and Rahe 1988) was used to assess colonization by *V. albo-atrum*. Alfalfa cuttings grown in styrofoam cups (200 cc) were uprooted, and the intact root systems were washed clean and excised from the stems. Roots that had curled at the bottoms of the cups were trimmed. Each root system was submerged for 2 min in approximately 250 ml of 1% NaOCl contained in a 125 mm Büchner funnel lined with filter paper. During the sterilization, the root system was spread out evenly with forceps, after which the NaOCl was drawn through the funnel with a vacuum of about 700 mm Hg. The roots were washed with sterile distilled water which was also drawn through the funnel. The filter paper with the adhering root system was

removed from the funnel with sterile forceps and inverted onto a 15 cm Petri dish containing water agar. The paper and the root system were pressed into the agar with a sterile bent glass rod. A small quantity of sterile distilled water was squirted onto the paper, and the paper was removed leaving the root system evenly spread on the agar. The plates were incubated at 22 °C in the light, and the origin of *V. albo-atrum* colonies growing from the roots was assessed at 3, 6, and 9 days after plating.

Evaluation of the root plating technique

Twenty cuttings each from B09 and P23 were rooted in distilled water. After 4 weeks, when the surviving cuttings had roots of ≥ 5 cm in length, the distal 1 cm portion of half of the roots on each cutting were cut with a sterile scalpel. The cuttings were inoculated as described, with either the *V. albo-atrum* spore suspension or distilled water. The root systems were then excised from the stems leaving about 3 mm of stem tissue attached, and plated as described. Assessments were made over time for colonies of *V. albo-atrum* on the cut and intact roots.

Root colonization quantification

Cuttings of B09 and P23 were rooted and grown in Metromix, inoculated after 6 weeks and repotted. Cuttings were rated for foliar symptoms and the whole root systems were plated at weekly intervals for an additional 6 weeks. Root systems for time zero were taken directly after inoculation; the remainder were kept in a completely randomized design in a growth chamber at 20 °C with 16 h/day photoperiod (230 $\mu\text{E}/\text{m}^2$). Twenty four replicate plants of each clone were assessed at each weekly interval.

The plated roots were assessed 3, 6 and 9 days after plating. The positions of colonies putatively identified as *V. albo-atrum* were marked on the Petri plates. The numbers of colonies expressing on the plates were recorded. A score, defined as the ratio of 'point source' to total number of colonies of *V. albo-atrum*, was given to each plate. A point source colony was defined as a colony originating from a length of < 1 mm of root; all other colonies were designated as 'continuous' colonies. After week 3, additional data on

colony types were obtained. The plates were photocopied after rating, and the length of root from which each colony originated was measured from the image. The average lengths of the colonies on each of the replicate plates were calculated and the mean average colony length value for each clone for each week was reported. For the purposes of these calculations point source colonies were considered to have a colony length of 1 mm.

Statistical Analysis

The two populations were analyzed using Chi square tests of the frequencies of symptom categories at each week of the experiment. The mean fresh weight comparisons were made using non paired sample t-tests. Comparisons between the number of colonies expressing on the roots of the two phenotypes over time were made using two-way analysis of variance. Individual point in time comparisons were made using non paired sample t-tests and Bonferroni interval tests to maintain confidence limits. Root assessments, average mean colony length measurements and mean symptom comparisons were carried out using two-way analysis of variance. Correlation comparisons were made between both the root assessment scales and average mean colony lengths (at all assessment dates) to the foliar symptom scale.

Results

Selection of resistant and susceptible phenotypes

Disease ratings in clonal populations derived from four mother phenotypes over 6 weeks are shown in figure 1. Based on these data, clones B09 and P23 were selected for further study. Figure 2 shows symptom development on inoculated and control plants of clones B09 and P23 for the two experimental trials used to select the phenotypes (A and B), and from the root plating experiment (C). Differences in foliar symptom severity in the two clones are apparent 2 to 3 weeks after inoculation. Chi square analysis of the frequencies of symptom categories in each clone revealed that the differences became significant ($P \leq 0.05$) at different times through to week six in the experimental trials.

Changes in fresh weights of the clones B09 and P23 are summarized in Table 1. In both trials, change in fresh weight was significantly reduced by inoculation in clone P23, but not in clone B09 (t-test, $P \leq 0.05$). Based on the foliar symptom and weight data, clones B09 and P23 were selected to represent resistant and susceptible phenotypes, respectively.

The results of the stem plating procedure demonstrated that *Verticillium* was recovered from stems of all inoculated plants. The plating procedure was also used to monitor the controls as any plant which was positive for *Verticillium* was removed from the analysis.

Assessment of invasiveness in roots

The only roots that yielded any *V. albo-atrum* colonies were those which had the tips excised, and were exposed to the *V. albo-atrum* conidial suspension. Undamaged roots exposed to the spore suspension were free of *V. albo-atrum* when plated, as were the control roots.

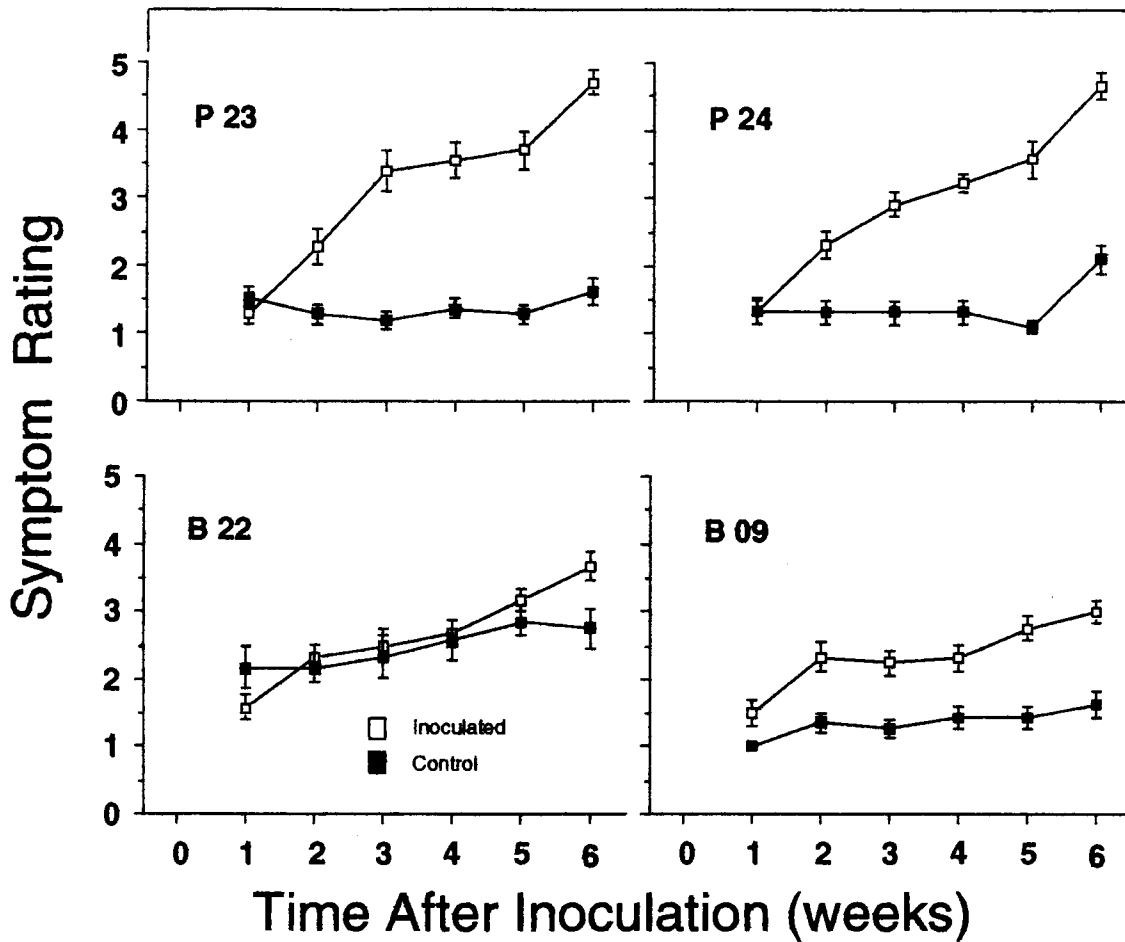


Figure 1. Foliar symptom development in four clonal populations of alfalfa following root inoculation with an alfalfa strain of *Verticillium albo-atrum*. The symptom ratings were based on the following scale: 1 = no symptoms, 5 = dead plant; and expressed as the mean \pm standard error of the ratings for 12 individually scored plants in each clone - treatment combination.

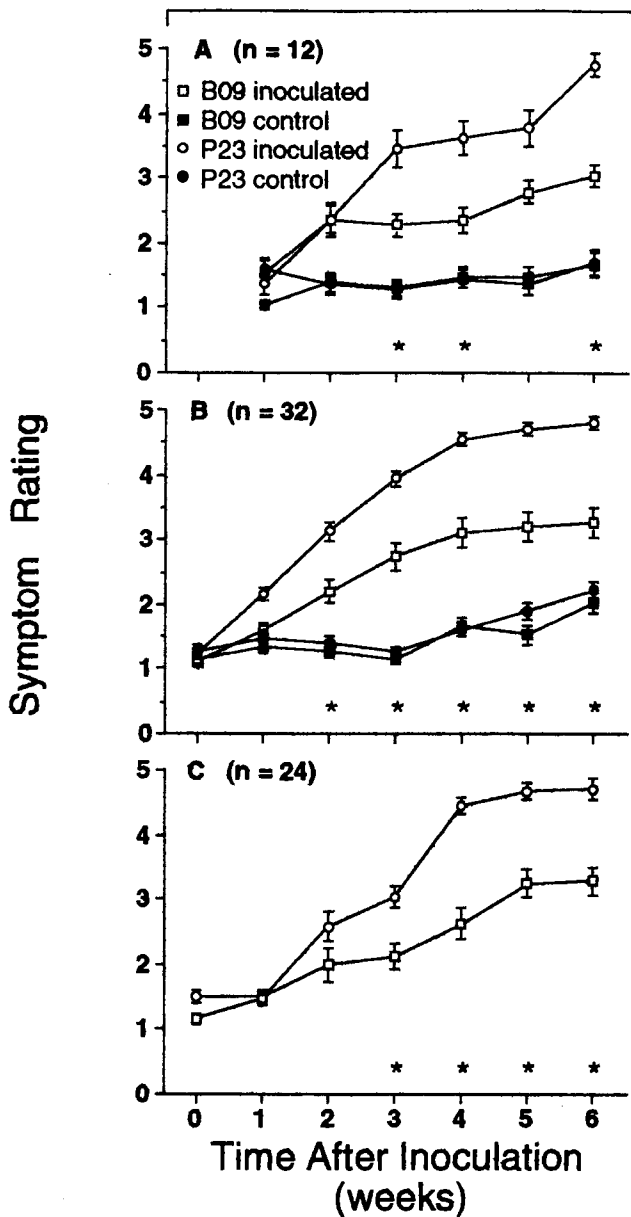


Figure 2. Foliar symptom development in phenotypically resistant (B09) and susceptible (P23) clonal populations of alfalfa following root inoculation with an alfalfa strain of *Verticillium albo-atrum*, in three trials (A, B and C). 1 = no symptoms, 5 = dead plant; expressed as the mean \pm standard error of the ratings. * indicates points in time when the two inoculated populations are significantly different ($P \leq 0.05$).

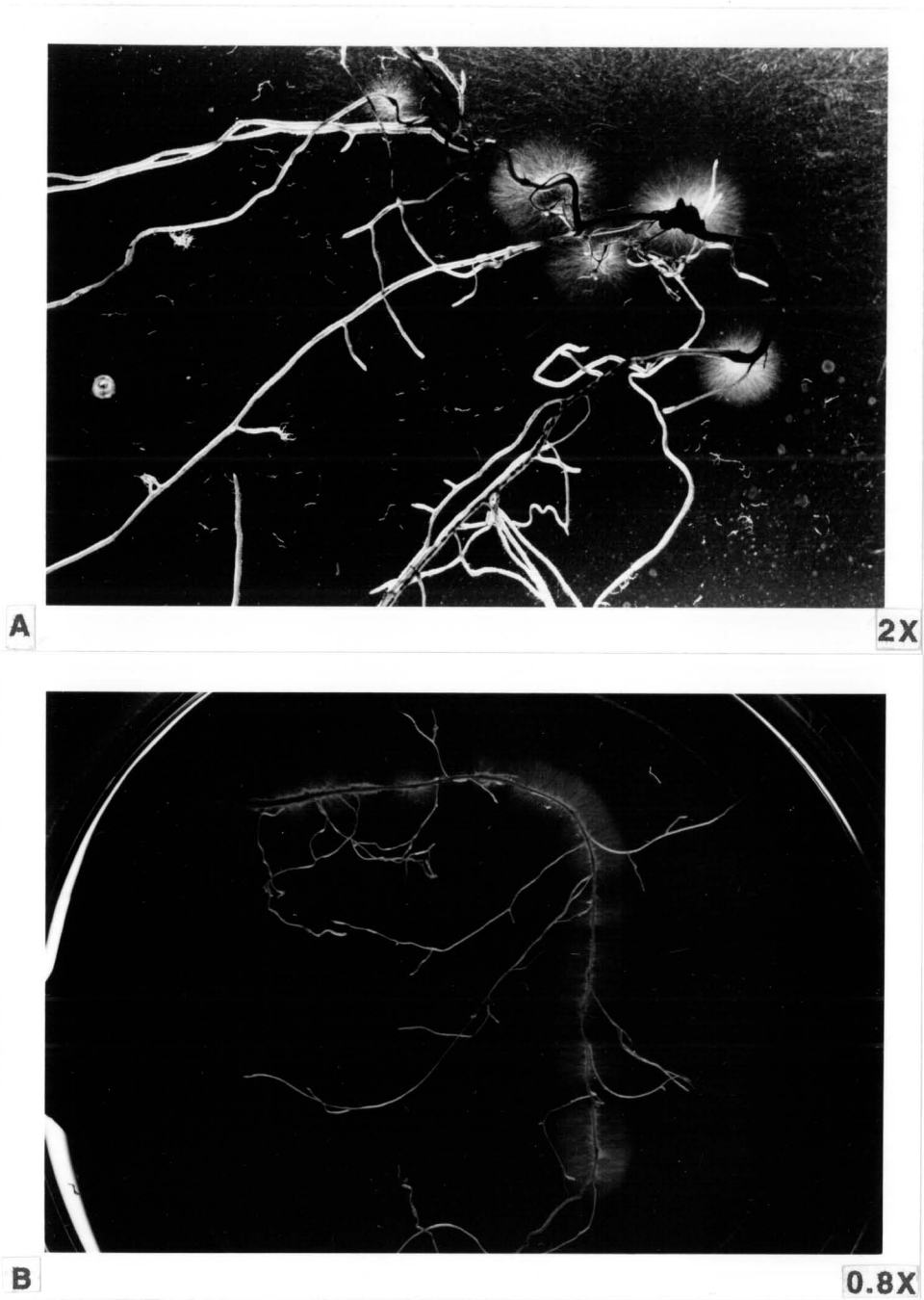
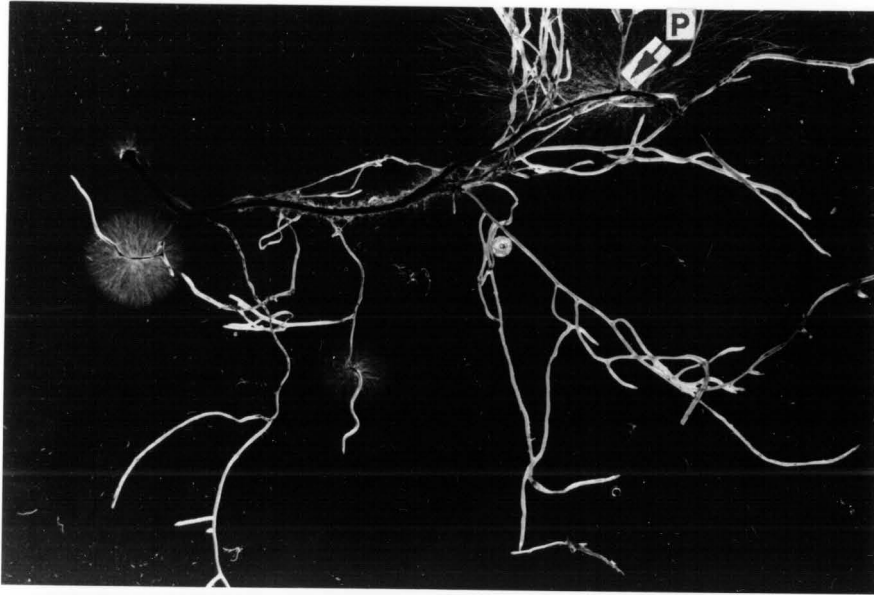
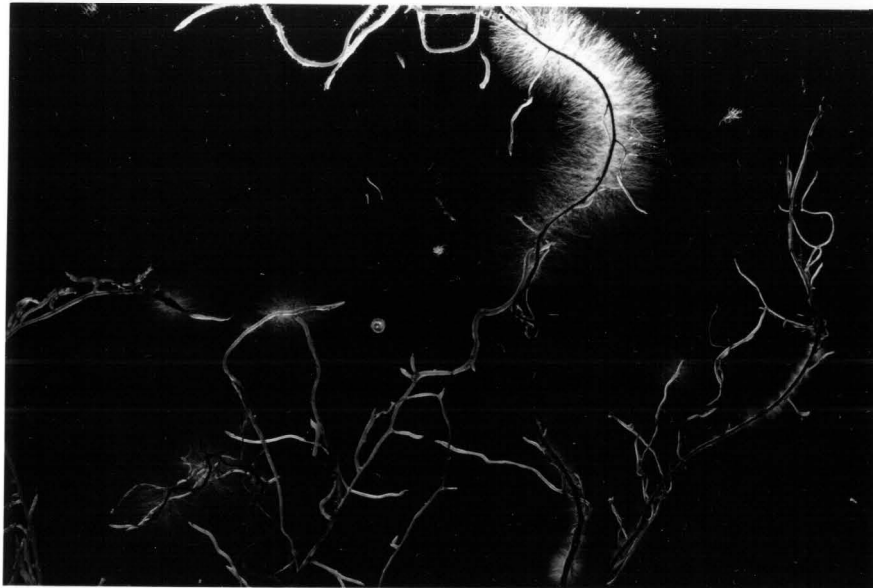


Figure 3. Photographs of plated alfalfa roots 6 days after plating, demonstrating both point source (A) and continuous (B) *Verticillium* colony types. The large colony emanating from the root crown in 'A' is *Pythium. sp.*, all other colonies are *Verticillium*.



1X



1X

Figure 4. Photographs of typical plated alfalfa roots (B09), 6 days after plating, both point source and continuous colonies of *Verticillium* being represented. All colonies are *Verticillium* except the colony denoted by a 'P' indicating *Pythium* sp.

Table 1. Effect of inoculation with an alfalfa strain of *Verticillium albo-atrum* on fresh weight (g) gain measured at 6 weeks after time of inoculation for individual plants of alfalfa clones B09 (resistant) and P23 (susceptible).

Clone	Treatment	
	Inoculated	Control
	Experiment 1 (n = 12)	
B09	5.37 ± 0.39 a ¹	5.90 ± 0.37 a
P23	1.00 ± 0.43 b	4.67 ± 0.39 c
	Experiment 2 (n = 32)	
B09	1.58 ± 0.39 a	2.24 ± 0.22 a
P23	-0.72 ± 0.16 b	2.97 ± 0.22 c

¹ Values followed by the same letter (vertical and horizontal comparisons within individual experiments only) are not significantly different ($P \leq 0.05$, t-test).

Figure 3 illustrates the point source and continuous colony types and figure 4 illustrates examples of the range of colonies seen on the plates.

Ratios of point source to total colonies emanating from the plated whole root systems of B09 and P23 at each week, are shown in figure 5. The lines were significantly different ($P \leq 0.01$) at each assessment period, with roots of B09, having a greater proportion of point source colonies than roots of P23. The trends of the lines representing the two clones are significantly different ($P \leq 0.05$) at the day 3 comparison, but not for the day 6 and 9 comparisons

Numbers of colonies expressing on the plates ranged from two to seven for the day 3 assessments, four to nine for the day 6 assessments and five to ten for the day 9 assessments. In general P23 had a higher number of colonies on the plates than B09.

Comparisons of the numbers of colonies expressing over time showed that the differences between the phenotypes were significant ($P \leq 0.05$, two way ANOVA) for the day 3 and day 6 assessments but not the day 9 assessments.

The mean colony length comparisons are shown in figure 6. The colonies of *V. albo-atrum* from roots of B09 were more restricted (shorter) than those on the roots of P23. The data were significantly different ($P \leq 0.01$, two way ANOVA) for both the day 6 and day 9 assessments and the trends of the lines are different ($P \leq 0.05$) for the day 6 assessments but not for the day 9 assessments. The comparative foliar symptom scores for the two clones were comparable in all the experiments (figure 2) and the two clones were significantly different ($P \leq 0.01$, two way ANOVA).

Probability values for comparisons of means for foliar symptoms, ratio of point source to total colonies, and colony length data, between B09 and P23 from 0 to 6 weeks after inoculation are shown in table 2. Weekly mean comparisons using either type of root assessment data did not show any particular point in time after inoculation when differences between the clones became consistently significantly different, whereas differences based on foliar symptom data were consistently different after week 3.

Correlation coefficients between the root colonization assessment data and the foliar symptom data showed that although both data sets distinguished clone B09 as resistant and P23 as susceptible, the root assessments were not highly correlated to the foliar assessment scale.

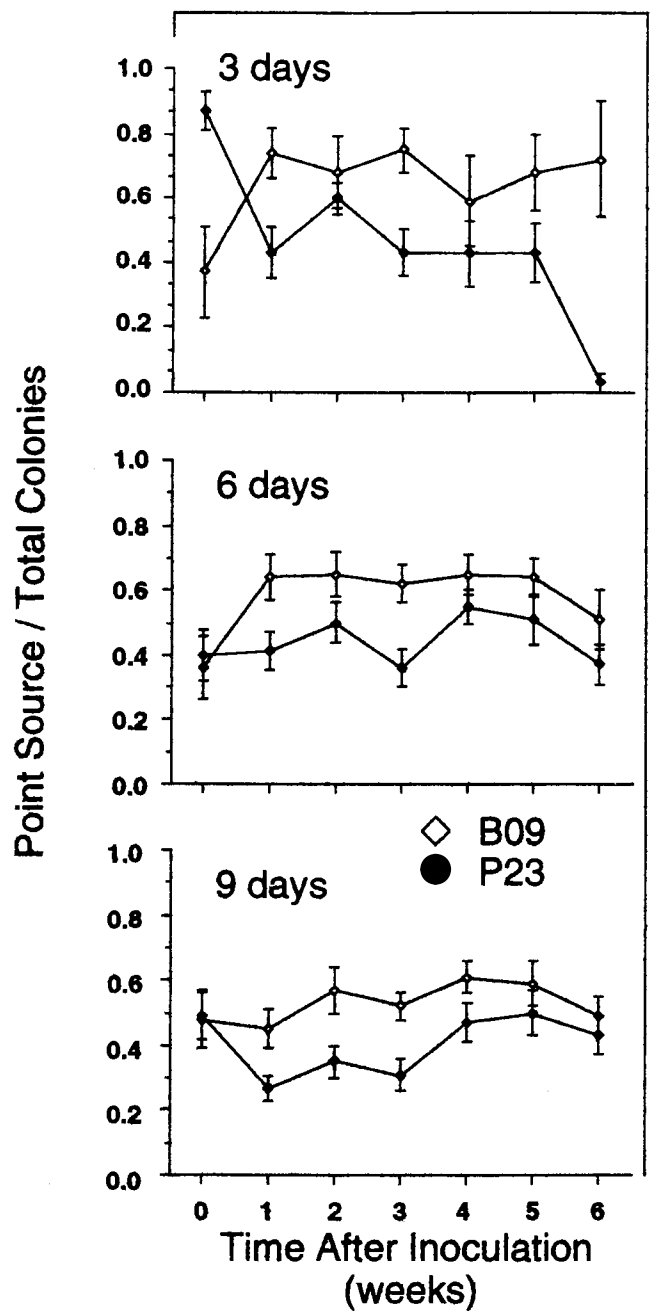


Figure 5. Ratios of point source to total colonies of *Verticillium albo-atrum* growing from roots of resistant (B09) and susceptible (P23) alfalfa expressed as the mean \pm standard error (n = 24), scored at 3, 6, and 9 days after plating of surface sterilized whole root systems harvested 0 - 6 weeks after inoculation.

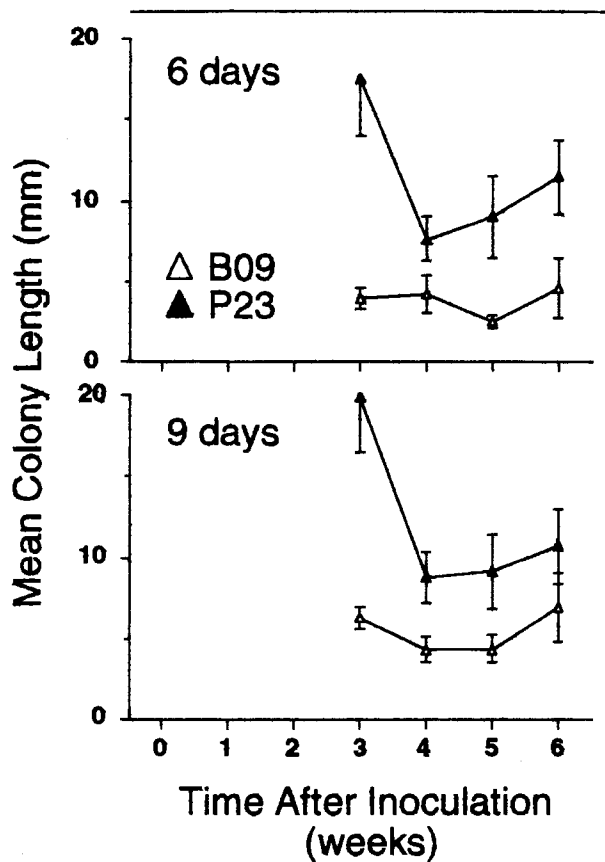


Figure 6. Mean values \pm standard error ($n = 24$) of the *Verticillium albo-atrum* colony lengths growing on roots of resistant (B09) and susceptible (P23) alfalfa plated in 15 cm diameter Petri dishes. Roots were scored at 0 - 6 weeks after inoculation. Colony lengths were defined as the length of root from which the colony emanated. Point source colonies were represented by a length of 1 mm.

Table 2. Two way ANOVA least square probability values from comparisons between *Verticillium albo-atrum* inoculated clonal alfalfa populations B09 and P23, from the foliar symptom ratings, point source/total colony root assessments and colony length measurements over all assessment periods for each week of the study.

Assessments

Week	Foliar symptoms	Day 3 colony assessments	Day 6 colony assessments	Day 9 colony assessments	Day 6 colony lengths	Day 9 colony lengths
0	0.2257	0.0004 *	0.6824	0.9868	-	-
1	0.8698	0.0064 *	0.0151 *	0.0306	-	-
2	0.0223	0.4463	0.1104	0.0388	-	-
3	0.0004 *	0.0025 *	0.0033 *	0.0117 *	0.0001 *	0.0001 *
4	0.0001 *	0.2539	0.3390	0.1255	0.2182	0.1090
5	0.0001 *	0.0479	0.1788	0.3363	0.0184 *	0.0881
6	0.0001 *	0.0001 *	0.1627	0.3396	0.0130 *	0.1773

* indicate a significant difference ($P \leq 0.05$) between the data for clones B09 and P23 as determined by Bonferroni interval test.

Discussion

The results from this study demonstrate that resistance can be detected in both the stems and roots of alfalfa plants infected with *Verticillium albo-atrum*. The same clonal populations of alfalfa plants which were distinguished as resistant and susceptible based on foliar symptom expression also were distinguished using the root plate assessments.

The results from the evaluation of the effectiveness of the root surface sterilization procedure showed that the *V. albo-atrum* colonies expressing on the plated roots originated from within the vascular system, since the sterilization would kill all surface borne contaminating propagules. The only roots from which *V. albo-atrum* was recovered were those that had been cut and inoculated. All plated stem pieces from *V. albo-atrum* treated cuttings had a colony developing from it, while stem pieces from control roots did not. Although the surface sterilization worked for all root surfaces, as demonstrated by no *V. albo-atrum* on treated uncut roots, it did not adequately disinfest the stem piece. This is not unexpected since the cut stem end from which the roots developed had not completely healed over at the time of testing, and was still an open wound.

The root plate assessment is based on the assumption that all *V. albo-atrum* colonies expressing on the plates originated from within the vascular system and not from conidia or other colony forming units residing on the root surfaces. Thus, the character of the expressing colony ie. point source or continuous, could be considered to be indicative of a mechanism of resistance operating within the plant.

By observing the distribution of *V. albo-atrum* colonies on all plated roots, it was apparent that colonization was discontinuous. Such a discontinuous colonization has been previously reported in stems, leaves, pedicels and peduncles of alfalfa plants (Pennypacker and Leath 1987, Huang et al 1985, Huang 1989), but not from the root system.

The distribution of colonies on the root systems of the resistant alfalfa phenotype was more discontinuous than that on the susceptible phenotype, and displayed a

significantly higher proportion of point source colonies than the susceptible plant. The resistant roots tended to have fewer colonies expressing on them. Also, the mean average colony length was shorter on the resistant phenotype. All of these differences, were evident at all the plate assessment periods. A higher proportion of point source colonies and shorter mean average colony lengths on the roots of the resistant clones could indicate more effective restriction of pathogen development within the vascular system.

Mean average colony length data was collected at week 3 since at this time the roots had considerably longer colony lengths, and the two alfalfa phenotypes generally were separable on the basis of foliar symptom expression. The root assessments were not highly correlated with the foliar symptom assessments.

Rating the root plates after 6 days was the most reliable. At day 3, it was too early and at day 9 assessments were more difficult because of the presence of surface contaminants eg. *Aspergillus* spp. and *Penicillium* spp. occurring on all plates.

Pythium spp. were fairly common on the plates and appeared to have their colony origins on the roots, but they did not interfere with the plate assessments as the mycelium did not obscure the root surface. Occasionally, a colony of *Fusarium* spp. was identified on the root, but this was easily distinguished from *Verticillium*.

The root assessment technique provided a measure of *Verticillium albo-atrum* invasiveness in the root systems of alfalfa plants, and identified resistant and susceptible host - pathogen responses which were consistent with the ratings for foliar symptoms. The root plating technique proved to be a valuable tool for observing the distribution of fungal colonization on root systems. The root colony ratio assessment is a relatively simple method of comparing *V. albo-atrum* invasiveness on the roots of alfalfa plants and making resistant and susceptibility determinations of these plants.

Chapter 2

Expansion of the Model System

The nature of resistance in alfalfa to *V. albo-atrum* is reported to be polygenic (Heale 1985), therefore it is logical to expect varying degrees of resistance to *V. albo-atrum*.

The previous research was successful in providing a model system to evaluate resistance. Its success relied on choosing the "right" plants to evaluate as large scale clonal populations. With the variety Barrier reported to contain 56% resistant plants to *V. albo-atrum* (Hanna and Huang 1987) and the variety Pacer reported to contain only 31% resistant plants (Busch and Smith 1981), the chances of identifying relatively resistant and susceptible phenotypes by screening a relatively small populations of plants were acceptable.

The objective of the research described in this chapter was to determine if a simple prescreening of individual seedlings would increase the probability of identifying a phenotype more resistant than B09 (the resistant phenotype in the model system).

Materials and Methods

Inoculum Production

The strain of *V. albo-atrum* used and the procedure for inoculum production was the same as described earlier.

Plant Material

Fifty alfalfa plants from each of the cultivars Barrier and Pacer were started from seed and maintained in a growth room at 20 °C with a 16 h day under fluorescent lights at an intensity of 240 $\mu\text{E}/\text{m}^2$. At week 6, each of the plants was cloned by taking two stem cuttings. These cuttings were rooted and grown in Metromix. The cuttings were maintained in a growth chamber with a 20 °C, 16 h day at 230 $\mu\text{E}/\text{m}^2$ and a 18 °C night. These clones represented a stock of *Verticillium* free material while the original seedlings were moved to the prescreening stage. At week 6 plus 2 days, the original seedlings were used in the prescreening inoculation procedure.

PreScreening

The prescreening process involved inoculating each of the fifty Barrier, and fifty Pacer plants originally started from seed. Six weeks after inoculation four plants (two Barrier, and two Pacer) were selected from the larger population to be advanced to the larger clonal population assessment. These plants were selected on the basis that they expressed minimal symptoms of susceptibility 6 weeks after inoculation with *V. albo-atrum*. The plants selected were designated B10 and B16 (from the variety Barrier), and P36 and P47 (from the variety Pacer). They were confirmed to be infected with *V. albo-atrum* by surface sterilizing 1 -1.5 cm long stem sections for 2 min in a 1% NaOCl solution, rinsing for 2 minutes in sterile distilled water, and then plating the segment on water agar.

The *Verticillium* free stock plants of phenotypes B10, B16, P36 and P47 were grown for six months in a growth chamber with a 20 °C, 16 hr day (230 μ E/m²), and a 18 °C night until plants of a suitable size to provide the required number of stem cuttings for larger clonal population screenings were obtained. At this time cuttings were taken, grown and maintained as described earlier. At 8 weeks the newly rooted cuttings were used for inoculation in the larger clonal population assessments.

Clonal Population Assessments

Forty eight clonal plants from each of the selected mother phenotypes were used in the larger clonal population assessments. Half of the plants from each clone were inoculated with the *V. albo-atrum* isolate, while the other half were used as controls. Plants were rated for disease symptoms at weekly intervals for 6 weeks following inoculation, using the same rating scale as previously described.

Inoculation and Screening of the Clonal Populations

The inoculation procedure for the clonal population assessment was similar to the prescreening inoculation procedure with the following exceptions. After washing, the plants were blotted dry and their wet weights were recorded. The control clones underwent a one h root soak in sterile distilled water instead of in the spore suspension. All the clones (24 repetitions per treatment) were replanted in styrofoam cups and arranged in a complete randomized design in a growth chamber with a 20 °C, 16 h day (230 μ E/m²) and a 18 °C night.

The clonal populations from phenotypes B10, B16, P36, and P47 were separated into control and inoculated groups and had their foliar disease symptoms rated at weekly intervals for 6 weeks following inoculation as previously described. Stem sections (1-1.5 cm long) from all plants (one/plant) were surface sterilized for 2 minutes in a 1% NaOCl

solution, rinsed for 2 minutes in sterile distilled water and then plated on water agar to confirm the presence of the fungus in the plants. The plates were incubated at 22 °C in the light. Control plants positive for *V. albo-atrum* were dropped from the analysis.

Statistical Analysis

The clonal populations were compared using Chi square tests of the frequencies of symptom categories at each week of the experiment. The mean fresh weight comparisons were made using non paired sample t-tests.

Results

The prescreening procedure resulted in the identification of 16 plants from the cultivar Barrier (32%), and six Pacer plants (12%) which were classified as resistant. Two Pacer plants, P36 and P47, and two Barrier plants, B10 and B16, were selected for clonal population assessment. Figure 7 shows disease progression as measured as mean symptom score for control and inoculated clonal populations of clones B16, B10, P47, and P36 over the 6 weeks of assessment. These progressions provide a useful visual comparison of disease progression over time which can help in determining if the week 6 scores are reasonable considering the overall trend of disease development for the plants.

The mean symptom scores of the inoculated treatments of the four new clones at week 6 are compared with the week 6 mean symptom score of clone B09 in table 3. The week 6 scores were used because the maximum symptom score in the inoculated plant occurred at this time. These results show that only one clone, B16 was significantly more resistant to *V. albo-atrum* than the previous most resistant plant B09. Two other clones, P36 and B10, were equally resistant, while clone P47 was significantly more susceptible.

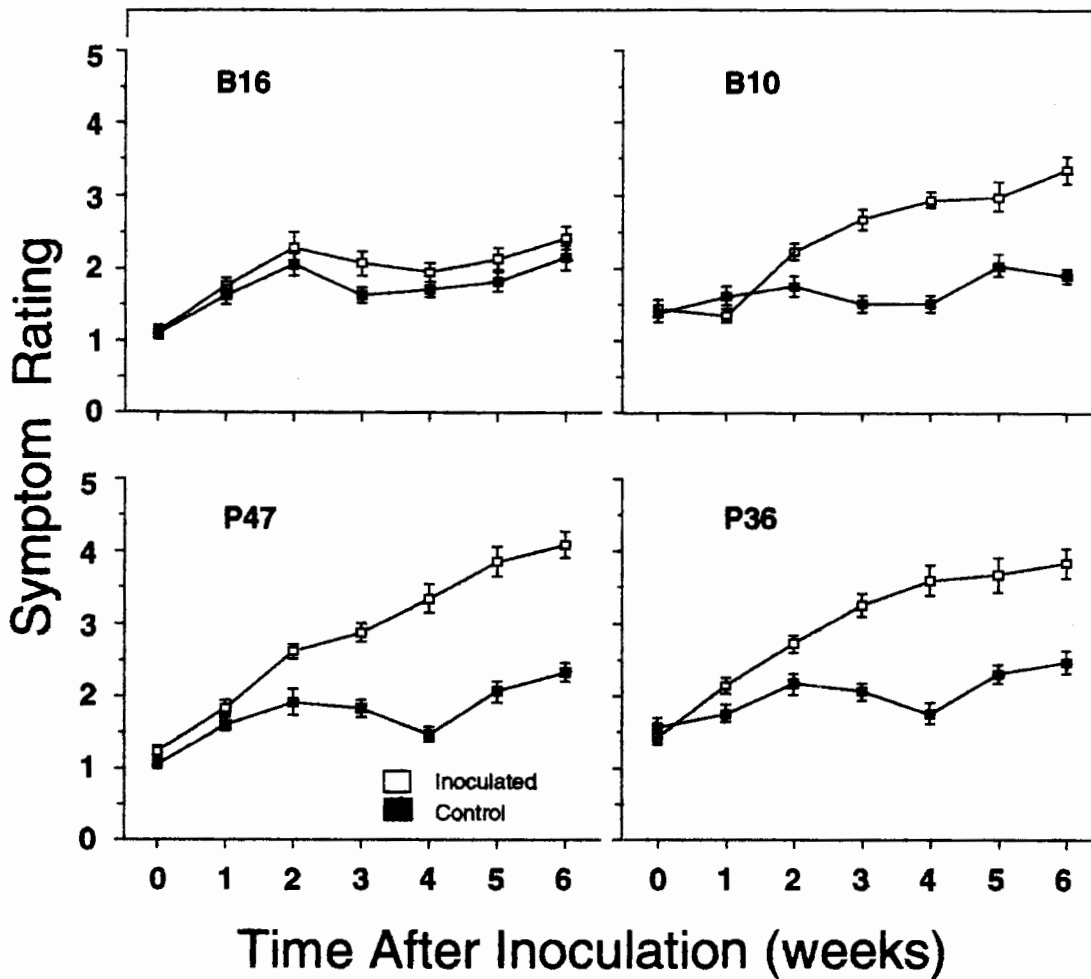


Figure 7. Foliar symptom development in four clonal populations of alfalfa following root inoculation with an alfalfa strain of *Verticillium albo-atrum*. The symptom ratings were based on the following scale: 1 = no symptoms, 5 = dead plant; and expressed as the mean \pm standard error of the ratings for 24 individually scored plants in each clone - treatment combination.

Table 3. Comparisons of week 6 mean foliar symptom scores for *Verticillium* inoculated alfalfa clonal populations of B10, B16, P36, and P47 to B09.

Clone	Week 6 Mean Symptom Score		
B09	3.22 ± 0.21	a ¹	n = 32
B10	3.36 ± 0.19	a	n = 24
B16	2.14 ± 0.15	b	n = 24
P47	4.13 ± 0.18	b	n = 24
P36	3.86 ± 0.21	a	n = 24

¹ Numbers followed by the same letter are not significantly different ($P \leq 0.05$, Chi square analysis). Only comparisons between cells were made, comparisons within a cell were not made

The mean wet weight gain of the inoculated populations of alfalfa plants B10, B16, P36, and P47 over the duration of the experiment are shown in table 4 where they are compared to the wet weight gain data from previous research on plant B09. These comparisons are consistent with the week 6 mean symptom score comparisons. Only clone B16 gained significantly more weight than B09, indicating increased resistance; plants B10 and P36 showed no difference in weight gain compared to plant B09 and plant P47 gained significantly less weight, indicating increased susceptibility.

Table 4. Effect of inoculation with an alfalfa strain of *Verticillium albo-atrum* on fresh weight (g) gain measured at 6 weeks after time of inoculation for individual plants of alfalfa clones B10, B16, P36, P47 compared to B09.

Clone	Mean Fresh Weight Gain (g)
B09	1.58 ± 0.39 a ¹ n = 32
B10	1.12 ± 0.23 a n = 24
B16	2.60 ± 0.28 b n = 24
P47	0.24 ± 0.20 b n = 24
P36	0.72 ± 0.22 a n = 24

¹ Numbers followed by the same letter are not significantly different ($P \leq 0.05$, t-test). Only between cell comparisons were made, comparisons within a cell were not made.

Based on the week 6 mean symptom score comparisons and the mean weight gain comparisons clones B10, P26 and P47 were judged undesirable for further study for incorporation into the model system.

Figure 8 shows the inoculated and control mean symptom score data from plant B16 superimposed on the inoculated and control mean symptom score data from plants B09 and P23 determined from previous research.

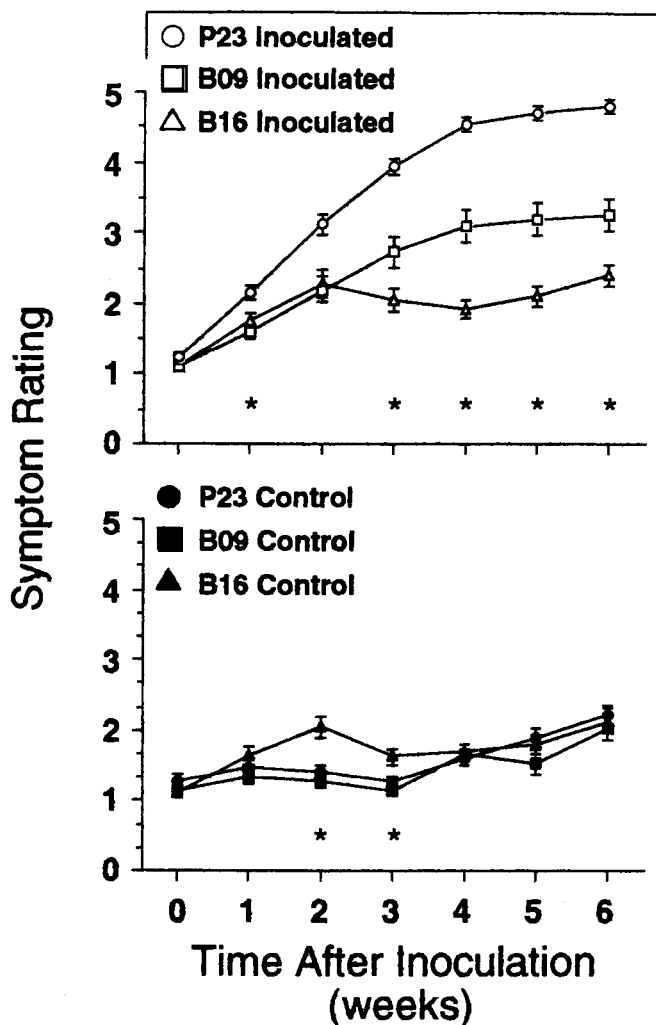


Figure 8. Comparison of foliar symptom development in inoculated (*Verticillium albo-atrum*) and control clonal populations of alfalfa plants B09, P23 and B16. Data for B09 and P23 are from experiment 2 reported in the main body of the thesis and represent the mean response of 32 clonal plants (\pm standard error). Data points for B16 represent the mean response of 24 clonal plants (\pm standard error). 1 = no symptoms, 5 = dead plant. * represent times when differences between all the inoculated populations of B09, P23 and B16 were significantly different ($P \leq 0.05$, Chi square analysis).

Previous research has shown that the differences between inoculated populations of B09 and P23 were significant at the 0.05 level (Chi square analysis) from weeks 2 through 6. The current comparisons show that the differences between inoculated populations of P23, B09 and B16 were consistently significant at the 0.05 level (Chi square analysis) from weeks 3 to 6. The differences between inoculated populations of P23 and B16 were consistently significant at the 0.05 level from week 1 through week 6. Differences between inoculated populations of B09 and B16 were significant (Chi square, $P \leq 0.05$) at weeks 1, 3, 4, 5, and 6, but not at week 2.

Differences between control populations of B09 and P23 were shown to be significant ($P \leq 0.05$) from time 0 to week 6. The differences between control populations of B09 and B16 were significant at weeks 2, 3 and 5 with plant B16 showing more symptoms. Differences between the control populations of B16 and P23 were significant only at weeks 2 and 3, again with clone B16 showing a higher symptom score, the differences were insignificant from weeks 4 to 6.

The symptom score for the inoculated population of clone B16 was consistently lower than for clones B09 and P23, but whenever differences between the control populations of B16 and B09 and P23 were significant, clone B16 always showed a higher symptom score.

Discussion

The four plants that were selected to carry forward from the prescreening process were classified as being more resistant (week 6 symptom scores of 2 or less) than B09 (week 6 average symptom score 3.22). However, when these selections were assessed using clonal populations, only one clone, B16 maintained this position of higher resistance when compared with clone B09. Clones B10 and P36 were found to be equally resistant to, and P47 was less resistant than B09. Based on these results, the prescreening process was 75% successful in identifying plants which were at least as resistant as plant B09. It was apparent with plant P47 that a low symptom score in the prescreening process does not guarantee that a plant will remain classified as resistant when it is assessed using larger clonal populations.

Plant B16 proved acceptable for consideration for incorporation into the model system, currently consisting of plants B09 and P23. Mean symptom score comparisons showed that plant B16 could be incorporated into the model system with a probability of significant differences between inoculated clonal populations from these plants, with plant B16 as most resistant, B09 as medium resistant, and P23 as susceptible from weeks 3 to 6 after inoculation.

References

- Arny, D.C. and C.R. Grau. 1985. Importance of verticillium wilt of alfalfa in North America. *Canadian Journal of Plant Pathology* 7: 187-190.
- Atkinson, T.G. 1983. What is the Problem? p. 1 *In* Verticillium wilt of alfalfa. H.C. Huang and T.G. Atkinson, (eds). Agriculture Canada Contribution 1982-8E.
- Beckman, C.H., W.C. Mueller, B.J. Tessier and N.A. Harrison. 1982. Recognition and callose deposition in response to vascular infection in fusarium wilt-resistant or susceptible tomato plants. *Physiological Plant Pathology* 20:1-10.
- Bishop, C.D. and R.M. Cooper. 1983. An ultrastructural study of root invasion in three vascular wilt diseases. *Physiological Plant Pathology* 22: 15-27.
- Bolton, J.L., B.P. Goplen and H. Baenziger, 1972. World Distribution and Historical Developments. pp 1-34. *In* Alfalfa Science and Technology. C.H. Hanson (ed), American Society of Agronomy. Madison, Wisconsin, USA.
- Bruehl, G.W. 1987. Soilborne Plant Pathogens. Macmillan Publishing Company, New York, USA.
- Busbice, T.H., R.R. Hill Jr. and H. L. Carnahan. 1972. Genetics and Breeding Procedures pp. 283-318. *In* Alfalfa Science and Technology. C.H. Hanson (ed), American Society of Agronomy. Madison, Wisconsin, USA.
- Busch, L.V. and E. Smith. 1981. Susceptibility of Ontario-grown alfalfa cultivars and certain *Medicago* species to *Verticillium albo-atrum*. *Canadian Journal of Plant Pathology* 3:169-172.
- Busch, L.V. and E.A. Smith. 1982. Reaction of a number of cultivated plants and weed species to an alfalfa isolate of *Verticillium albo-atrum*. *Canadian Journal of Plant Pathology* 4: 266-268.
- Busch, L.V., B.R. Christie, E.A. Smith and G. Boland. 1985. Testing alfalfa cultivars for resistance to an alfalfa strain of *Verticillium albo-atrum*. *Canadian Journal of Plant Pathology* 7:203-205.
- Carder, J.H., R.C. Hignett and T.R. Swinburne. 1987. Relationship between the virulence of hop isolates of *Verticillium albo-atrum* and their *in vitro* secretion of cell-wall degrading enzymes. *Physiological and Molecular Plant Pathology* 31:441-452.
- Christen, A.A. and R.N. Peaden. 1981. Verticillium wilt in alfalfa. *Plant Disease* 65:319-321.
- Christen, A.A. and R.C. French. 1982. Growth and pathogenicity of alfalfa strain of *Verticillium albo-atrum*. *Plant Disease* 66:416-418.

- Christen, A.A., R.N. Peadar, G.P. Harris and J.B. Heale. 1983. Virulence of North American and European Isolates of *Verticillium albo-atrum* on alfalfa cultivars. *Phytopathology* 73:1051-1054.
- Christie, B.R., Y.A. Papadopoulos and L.V. Busch. 1985. Genetics and breeding for resistance to verticillium wilt in alfalfa. *Canadian Journal of Plant Pathology* 7:206-210.
- Correll, J.C., T.R. Gordon and A.H. McCain. 1988. Vegetative compatibility and pathogenicity of *Verticillium albo-atrum*. *Phytopathology* 78:1017-1021.
- Dixon, G.R., C.D. Kershaw and E.A. Hunter. 1989. Crop yields from lucerne (*Medicago sativa*) cultivars displaying gradations in resistance to wilt (*Verticillium albo-atrum*). *Journal of Agricultural Science, Cambridge* 112:387-394.
- Durrands, P.K. and R.M. Cooper. 1988a. Selection and characterization of pectinase-deficient mutants of the vascular wilt pathogen *Verticillium albo-atrum*. *Physiological and Molecular Plant Pathology* 32:343-362.
- Durrands, P.K. and R.M. Cooper. 1988b. The role of pectinases in vascular wilt disease as determined by defined mutants of *Verticillium albo-atrum*. *Physiological and Molecular Plant Pathology* 32:363-371.
- Elango, D.J., J. Robb, G. Newcombe and L.V. Busch. 1986. Growth pouch technique for the observation of cellular interactions between alfalfa seedling roots and *Verticillium albo-atrum*. *Canadian Journal of Plant Pathology* 8:78-84.
- Flood, J. and J.M. Milton. 1982. Studies on the incompatible and compatible interactions between lucerne leaves and *Verticillium albo-atrum*. *Physiological Plant Pathology* 21:97-104.
- Flood, J., F.Z. Khan, and J.M. Milton. 1978. The role of phytoalexins in *Verticillium* wilt of lucerne (*Medicago sativa*). *Annals of Applied Biology* 89:287-378.
- Gäumann, E. 1958. The mechanisms of fusaric acid injury. *Phytopathology* 48:670-686.
- Gerik, J.S. and O.C. Huisman. 1988. Study of field-grown cotton roots infected with *Verticillium dahliae* using an immunoenzymatic staining technique. *Phytopathology* 78:1174-1178.
- Graham, J.H., R.N. Peadar and D.W. Evans. 1977. *Verticillium* wilt of alfalfa found in the United States. *Plant Disease Reporter* 61:337-340.
- Graham, J.H., F.I. Frosheiser, D.L. Stuteville and D.C. Erwin. 1979. A compendium of alfalfa diseases. The American Pytopathological Society, St. Paul, Minnesota.

- Grau, C.R., S.L. Nygaard, D.C. Arny and P.A. Delwiche. 1991. Comparison of methods to evaluate alfalfa cultivars for reaction to *Verticillium albo-atrum*. *Plant Disease* 75:82-85.
- Hanna, M.R. and H.C. Huang. 1987. Barrier alfalfa. *Canadian Journal of Plant Science* 67:827-830.
- Harper, A.M. and H.C. Huang. 1984. Contamination of insects by the plant pathogen *Verticillium albo-atrum* in an alfalfa field. *Environmental Entomology* 13:117-120.
- Harper, A.M., H.C. Huang and G.C. Kozub. 1988. Survival of *Verticillium albo-atrum* on insect bodies and in insect feces at various temperatures. *Journal of Economic Entomology* 81:1799-1802.
- Harvey, A.M. and T.G. Atkinson. 1983. Why be concerned? pp 2-5 *In* *Verticillium wilt of alfalfa*. H.C. Huang and T.G. Atkinson, (eds). Agriculture Canada Contribution 1982-8E.
- Hawthorne, B.T. 1987. Qualitative and quantitative assessments of the reaction of lucerne plants to verticillium wilt. *New Zealand Journal of Agricultural Research* 30:349-359.
- Heale, J.B. 1985. *Verticillium wilt of alfalfa, background and current research*. *Canadian Journal of Plant Pathology* 7:191-198.
- Heale, J.B. and D.P. Gupta. 1972. Mechanism of vascular wilting induced by *Verticillium albo-atrum*. *Transactions British Mycological Society* 58:19-28.
- Heale, J.B. and I. Isaac. 1963. Wilt of lucerne caused by species of *Verticillium* IV. Pathogenicity of *V. albo-atrum* and *V. dahliae* to lucerne and other crops; spread and survival of *V. albo-atrum* in soil and in weeds; effect upon lucerne production. *Annals of Applied Biology* 52:439-451.
- Heinrichs, D.H., S. Bingfors, L.V. Crowder and R.H.M. Lange. 1972. Highlights of Research Around the World. pp. 737-774. *In* *Alfalfa Science and Technology*. C.H. Hanson (ed), American Society of Agronomy. Madison, Wisconsin, USA.
- Howard, R.J. 1985. Local and long-distance spread of *Verticillium* species causing wilt of alfalfa. *Canadian Journal of Plant Pathology* 7:199-202.
- Huang, H.C., A.M. Harper, E.G. Kokko and R.J. Howard. 1981. Aphid transmission of *Verticillium albo-atrum* to alfalfa. *Canadian Journal of Plant Pathology* 5:141-147.
- Huang, H.C. and M.R. Hanna. 1983. How can *Verticillium* wilt be controlled?. pp. 13-16. *In* *Verticillium wilt of alfalfa*. H.C. Huang and T.G. Atkinson, (eds). Agriculture Canada Contribution 1982-8E.

- Huang, H.C. and K.W. Richards. 1983. *Verticillium albo-atrum* contamination on leaf pieces forming cells for the alfalfa leafcutter bee. *Canadian Journal of Plant Pathology* 5:248-250.
- Huang, H.C. and A.M. Harper. 1985. Survival of *Verticillium albo-atrum* from alfalfa in feces of leaf-chewing insects. *Phytopathology* 75:206-208.
- Huang, H.C., M.R. Hanna and E.G. Kokko. 1985. Mechanisms of seed contamination by *Verticillium albo-atrum* in alfalfa. *Phytopathology* 75:482-488.
- Huang, H.C. and E.G. Kokko. 1985. Infection of alfalfa pollen by *Verticillium albo-atrum*. *Phytopathology* 75:859-865.
- Huang, H.C., K.W. Richards and E.G. Kokko. 1986a. Role of the leafcutter bee in dissemination of *Verticillium albo-atrum* in alfalfa. *Phytopathology* 76:75-79.
- Huang, H.C., R. Hironaka and R.J. Howard. 1986b. Survival of *Verticillium albo-atrum* in alfalfa tissue buried in manure or fed to sheep. *Plant Disease* 70:218-221.
- Huang, H.C. 1989. Distribution of *Verticillium albo-atrum* in symptomed and symptomless leaflets of alfalfa. *Canadian Journal of Plant Pathology* 11:235-241.
- Ireland, K.F. and K.T. Leath. 1987. Potential of using culture filtrates from *Verticillium albo-atrum* to evaluate alfalfa germ plasm for resistance to *Verticillium* wilt. *Plant Disease* 71:900-903.
- Isaac, I. 1949. A comparative study of pathogenic isolates of *Verticillium*. *Transactions British Mycological Society* 32:137-157.
- Isaac, I. 1957. Wilt of Lucerne caused by species of *Verticillium*. *Annals of Applied Biology* 45:550-558.
- Isaac, I. and A.T.E. Lloyd. 1957. Wilt of lucerne caused by species of *Verticillium* II. Seasonal cycle of disease; range of pathogenicity; host-parasite relations; effects of seed dressings. *Annals of Applied Biology* 47:673-684.
- Jimenez-Díaz, R.M. and R.L. Millar. 1986. Lack of systemic colonization of alfalfa plants after inoculation of uninjured leaves with conidia of *Verticillium albo-atrum*. *Plant Disease* 70:509-515.
- Jimenez-Díaz, R.M. and R.L. Millar. 1988. Sporulation on infected tissues, and presence of airborne *Verticillium albo-atrum* in alfalfa fields in New York. *Plant Pathology* 37:64-70.
- Khan, F.Z. and J.M. Milton. 1975. Phytoalexin production by lucerne (*Medicago sativa* L.) in response to infection by *Verticillium*. *Physiological Plant Pathology* 7:179-187.

- Khan, F.Z. and J.M. Milton. 1978. Phytoalexin production and the resistance of lucerne (*Medicago sativa* L.) to *Verticillium albo-atrum*. *Physiological Plant Pathology* 13:215-221.
- Khan, F.Z. and J.M. Milton. 1979. Some factors affecting the production of medicarpin and sativan by lucerne leaflets in response to *Verticillium albo-atrum*. *Physiological Plant Pathology* 14:11-17.
- Kotz, S. and N.L. Johnson.(eds) 1982. *Encyclopedia of Statistical Sciences* Vol 1. pp. 294-300. John Wiley and Sons, New York, USA.
- Latunde-Dada, A.O., R.A. Dixon and J.A. Lucas. 1987. Induction of phytoalexin biosynthetic enzymes in resistant and susceptible lucerne callus lines infected with *Verticillium albo-atrum*. *Physiological and Molecular Plant Pathology* 31:15-23.
- Latunde-Dada, A.O. and J.A. Lucas. 1988. Somaclonal variation and resistance to *Verticillium* wilt in lucerne, *Medicago sativa* L., plants regenerated from callus. *Plant Science* 58:111-119.
- Lévesque, C.A. and J.E. Rahe. 1988. A new technique for studying fungal colonization of roots. *Canadian Journal of Plant Pathology (Abstr.)* 10:368.
- McGeary, F.M. and A.C. Hastie. 1982. Hybridization of *Verticillium albo-atrum* strains from tomato and lucerne. *Physiological Plant Pathology* 21:437-444.
- Michail, S.H. and A.J.H. Carr. 1966. Use of culture filtrates as a rapid technique for screening lucerne for resistance to *Verticillium albo-atrum*. *Transactions British Mycological Society* 49:133-138.
- Nachmias, A., V. Buchner and Y. Burstein. 1985. Biological and immunochemical characterization of a low molecular weight phytotoxin isolated from a protein-lipopolysaccharide complex produced by a potato isolate of *Verticillium dahliae* Kleb. *Physiological Plant Pathology* 26:43-55.
- Nachmias, A., V. Buchner, L. Tsrer, Y. Burnstein and N. Keen. 1987. Differential phytotoxicity of peptides from culture fluids of *Verticillium dahliae* races 1 and 2 and their relationship to pathogenicity of the fungi on tomato. *Phytopathology* 77:506-510.
- Newcombe, A.G., Y.A. Papadopoulos, J. Robb and B.R. Christie. 1989. The colonization ratio: a measure of pathogen invasiveness and host resistance in *Verticillium* wilt of alfalfa. *Canadian Journal of Botany* 67:365-370.
- Newcombe, G. and J. Robb. 1988. The function and relative importance of the vascular coating response in highly resistant, moderately resistant and susceptible alfalfa infected by *Verticillium albo-atrum*. *Physiological and Molecular Plant Pathology* 33:47-58.

- Newcombe, G. and J. Robb. 1989a. The chronological development of a lipid - to - suberin response at *Verticillium* trapping sites in alfalfa. *Physiological and Molecular Plant Pathology* 34:55-73.
- Newcombe, G. and J. Robb. 1989b. An improved method of determining the colonization ratio in *Verticillium*-infected alfalfa plants. *Canadian Journal of Plant Pathology* 11:60-64.
- Nielsen, H.M., and Andreassen, B. 1975. *Verticillium albo-atrum* in lucerne. II. The effect of selection and the hereditary variation in resistance. pp. 79-90 *In* K. Vet. Landbohøjsk. Arsskr.
- Panton, C.A. 1965. The breeding of lucerne, *Medicago sativa* L., for resistance to *Verticillium albo-atrum* Rke. and Berth. I. Preliminary studies on the effectiveness of selection and investigations on methods for inducing symptom development and facilitating selection in early seedling stage. *Acta Agriculturae Scandinavica* 15:85-100.
- Peadar, R.N., R.G. Gilbert and A.A. Christen. 1985. Control of *Verticillium albo-atrum* on alfalfa. *Canadian Journal of Plant Pathology* 7:211-214.
- Pennypacker, B.W. and K.T. Leath. 1983. Dispersal of *Verticillium albo-atrum* in the xylem of alfalfa. *Plant Disease* 67:1226-1229.
- Pennypacker, B.W., K.T. Leath and R.R. Hill Jr. 1985. Resistant alfalfa plants as symptomless carriers of *Verticillium albo-atrum*. *Plant Disease* 69:510-511.
- Pennypacker, B.W. and K.T. Leath. 1986. Anatomical response of a susceptible alfalfa clone infected with *Verticillium albo-atrum*. *Phytopathology* 76:522-527.
- Pennypacker, B.W., K.T. Leath and R.R. Hill Jr. 1988. Growth and flowering of resistant alfalfa infected by *Verticillium albo-atrum*. *Plant Disease* 72:397-400.
- Pennypacker, B.W., K.T. Leath and R.R. Hill Jr. 1990. Growth and physiological response of resistant alfalfa clones infected with *Verticillium albo-atrum*. *Phytopathology* 80:247-253.
- Robb, J., D.A. Powell and P.F.S. Street. 1987. Time course of wall-coating secretion in *Verticillium*-infected tomatoes. *Physiological and Molecular Plant Pathology* 31:217-226.
- SAS Institute Inc. 1985 *SAS User's Guide:Basics, Version 5 Edition*. Cary, N.C.:SAS Institute Inc..
- SAS Institute Inc. 1985 *SAS User's Guide:Statistics, Version 5 Edition*. Cary, N.C.:SAS Institute Inc..
- Selman, I.W. and W.R. Buckley. 1959. Factors affecting the invasion of tomato roots by *Verticillium albo-atrum*. *Transactions British Mycological Society* 42:227-234.

- Talboys, P.W. 1964. A concept of the host-parasite relationship in *Verticillium* wilt diseases. *Nature* 202:361-364.
- Viands, D.R. 1985. Comparison of 'Maris Kabul' with 'Vertus' alfalfa for resistance to *Verticillium* wilt. *Crop Sci.* 25:1096-1100.
- Yorston, J.M. 1983. What is *Verticillium* wilt pp. 6-12. In *Verticillium wilt of alfalfa*. H.C. Huang and T.G. Atkinson, (eds). Agriculture Canada Contribution 1982-8E.