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Abstract

Grünwald, Niklaus J. and Coyne, Clarice J., eds. 2003. Proceedings of the Second International Aphanomyces Workshop, June 17-18, 2003. U.S. Department of Agriculture, Agricultural Research Service.

This report comprises papers presented at the Second International Aphanomyces Workshop, June 17-18, 2003, Pasco, Washington. These proceedings summarize scientific contributions from the workshop on the genus *Aphanomyces* that emphasized the importance of these pathogens on legumes and sugar beets. The workshop covered a broad range of subject matter including disease management, host-parasite interactions, epidemiology, population genetics, breeding for resistance, and economic impact. These proceedings also include a bibliography on the genus *Aphanomyces*.

Keywords: *Aphanomyces*, *Aphanomyces euteiches*, *Aphanomyces cochlioides*, root rot, legumes, sugar beets, crop pathology, peas, alfalfa, phytopathogen

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Foreword

Aphanomyces is an economically important genus of Oomycete pathogens that affect a wide range of hosts including on the one hand fish and crayfish and on the other hand crops such as alfalfa, bean, lentil, pea and sugarbeet. These proceedings summarize scientific contributions from a recently held workshop on the genus *Aphanomyces* that emphasized the importance of these pathogens on legumes and sugarbeet.

One recurring question raised during the second international Aphanomyces workshop was when the first Aphanomyces workshop took place. The first workshop was held in Le Rheu, France in 2002 following the initiative of our colleagues from Institut National de la Recherche Agronomique (INRA), Unité Mixte de Recherche. The first workshop served to remind us all how important Aphanomyces root rot is on pea. This workshop included breeders and plant pathologists working on pea and focusing solely on *A. euteiches* from the USDA, INRA and private industry. The first workshop was organized and sponsored by INRA, France.

The Second International Aphanomyces Workshop was held in Pasco, WA, on June 17 and 18, 2003. This workshop was expanded to include scientists from private industry, university, government, and international institutions. The workshop was also expanded in scope in that two important *Aphanomyces* species, namely *A. euteiches* and *A. coccineus*, as well as several hosts were included, namely those causing Aphanomyces root rot on alfalfa, bean, pea and sugarbeet. The workshop covered a broad range of subject matter including disease management, host-parasite interactions, epidemiology, population genetics, breeding for resistance, and economic impact. Over forty participants coming from France, New Zealand, California, Idaho, Illinois, North Dakota, Minnesota, Montana, Ohio, Oregon, Washington, and Wisconsin attended the workshop.

The workshop included a discussion session that provided critical observations on the need for more research and funding for the genus *Aphanomyces*. Participants agreed on the fact that Aphanomyces remains one of the most important yield-limiting factors in production of legumes and sugarbeet. Yet, the number of scientists working on this pathogen group has shrunken significantly. This is also reflected by the fact that the last comprehensive treatise on the genus Aphanomyces was published by Papavizas and Ayers in 1974 and has not been updated since. These proceedings also contain a bibliography on the genus *Aphanomyces* assembled to serve as a handy reference for the *Aphanomyces* community.

It is hoped that this workshop will renew interest in addressing the serious problems that the pathogens in the genus *Aphanomyces* cause.

Niklaus J. Grünwald & Clarice Coyne

Workshop Organizers and Proceedings Editors

Agenda

Monday, June 16, 2003

18:00-20:30 Welcome Reception

Tuesday, June 17

7:00-8:00 Breakfast

Welcome

8:00 Welcome – Niklaus Grunwald, USDA ARS
8:05 USDA-INRA Collaboration & Welcome – Dr. Rick Bennett, USDA ARS
8:15 The Vegetable and Forage Crop Research Unit, Prosser, WA – Dr. Ashok Alva, USDA ARS

Section I: Crop Pathology & Economic Impact

Moderator: Carol Windels

8:30 Niklaus Grunwald, USDA ARS: The biology of the genus *Aphanomyces*
9:00 Craig Grau, University of Wisconsin: Impact of Aphanomyces root rot on legumes in the Midwest
9:30 Bernard Tivoli, INRA, France: The Impact of Aphanomyces root rot in France

10:00 Coffee break

10:15 Carol Windels, University of Minnesota: Aphanomyces root rot of sugar beet
10:45 Chuck Martin, Del Monte: An industry perspective on Aphanomyces root rot on fresh peas
11:15 Dean Malvick, University of Illinois: *Aphanomyces* associated with alfalfa in the Midwestern USA

12:00-13:00 Lunch

Section II: Epidemiology, population genetics, and host-parasite interactions
Moderator: John Weiland

13:00	Anne Moussart, INRA, France: Pathology research programs in France to control Aphanomyces root rot
13:20	John Weiland, USDA ARS: Search for Virulence Determinants in the phytopathogenic *Aphanomyces*
13:40	Sophien Kamoun, Ohio State University: Oomycete genomics
14:10	George Vandemark, USDA ARS: Real time PCR assay for examining resistance to *A. euteiches* and studying microbial population dynamics in mixed plant infections
14:30	Coffee break
14:45	Niklaus Grunwald, USDA ARS: Within field phenotypic and genotypic diversity in *Aphanomyces euteiches*
15:05	Tom Darnell, Oregon State University: The Impact of Aphanomyces root rot in the Pacific Northwest
15:25	short break

15:30-18:00 Roundtable discussion
Moderator: Craig Grau

Objective: to stimulate discussion and information exchange regarding:
- practical issues (protocols, methods, tricks and tips); website?
- the future of *Aphanomyces* research (genomics, QTL-mapping, host-pathogen interactions, evolution, phylogeography, disease management, etc.)
- book on Aphanomyces

18:00-19:30 Dinner

Wednesday, June 18

7:00-8:00 Breakfast

Section III: Plant Breeding

Moderator: Norm Weeden

8:00 Rebecca McGee, General Mills, Inc., US: Breeding for resistance Aphanomyces root rot in pea: an industry perspective

8:20 Norm Weeden, Montana State University: Genetic factors affecting Aphanomyces tolerance and root growth in the pea line MN313

8:40 Alain Baranger & Marie-Laure Pilet-Nayel, INRA, France: Genetics of partial resistance/tolerance to Aphanomyces root rot

9:10 Clare Coyne, USDA ARS: Application of SNPs in identifying new positive alleles for genetic resistance to Aphanomyces

9:30 Kevin McPhee, USDA ARS: Challenges in breeding grain legumes for resistance to fungal pathogens

9:50 Mark Smith, Pioneer Hi Bred Intl.: Breeding for Aphanomyces tolerance in alfalfa: An industry perspective

10:10 Coffee break

10:20 Frédéric Muel, French Grower Cooperative, GSP, France: GSP breeding program for resistance to Aphanomyces root rot

10:40 Margaret Rekoske, Betaseed, Shakopee, MN: Breeding for Aphanomyces tolerance in sugarbeet: An industry perspective

11:00 Gail Timmerman-Vaughan, Crop & Food Research Institute, New Zealand: Association mapping to study the genetics of Aphanomyces root rot resistance

11:20 Wrap-up session

12:00-13:00 Lunch

<u>Fieldtrips, afternoon of June 18:</u>

13:15 Leave for Prosser

14:00 The Irrigated Agriculture Research and Extension Center, Washington State University, Prosser, WA
The USDA Legume pathology and alfalfa breeding programs, Prosser
The USDA Roza pea and bean root rot nurseries, Prosser

5:00 Tour Hogue Cellars winery

6:30 Adjourn; return to Red Lion Hotel

Section I: Crop Pathology & Economic Impact

The biology of the genus *Aphanomyces*

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The genus *Aphanomyces*, is among the smaller and less frequently encountered genera within the Saprolegniaceae (Scott 1961; Webster 1980). This group of organisms is most closely related to the golden brown algae and has been placed within the stramenopiles (Leipe et al. 1994).

Within the family Saprolegniaceae, containing among others the genera *Saprolegnia, Dictyuchus,* and *Achlya,* the genus *Aphanomyces* constitutes an ancestral group (Figure 1, 2) (Cooke et al. 2000; Leclerc, Guillot, and Deville 2000).

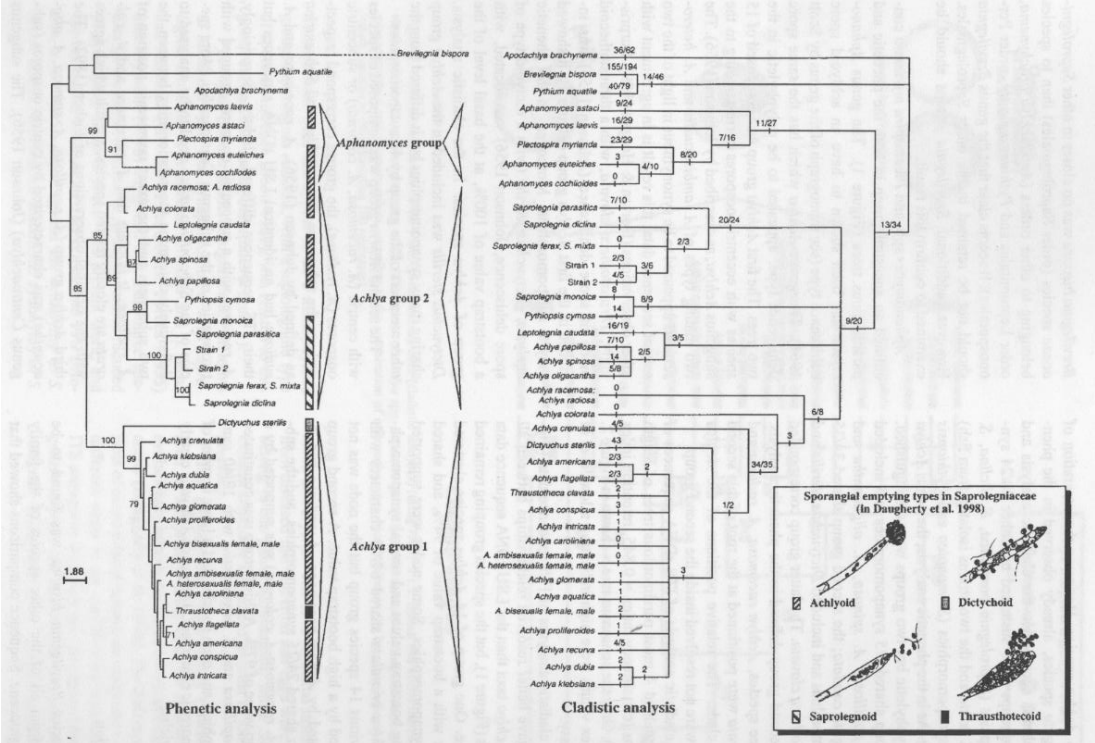

Figure 1. Distance tree (phenetic analysis) constructed by the neighbor-joining method from the 622 aligned positions of LSU rDNA (left). Numbers on the branches indicate the bootstrap values superior to 70% (based on 1000 replicates). Scale bar = 1.88% divergence. The strict consensus trees of the 4279 most parsimonious trees (cladistic analysis) (using PAUP 3.1.1., heuristic search, gaps = fifth state), from the 622 aligned positions of LSU rDNA (right). Numbers on the branches indicate the minimal/maximal number of synapomorphies and autapomorphies according to the optimization of informative sites.

Figure 1. Distance and consensus trees constructed based on LSU rDNA. Figure taken from Leclerc et al. (2000).

The genus *Aphanomyces* has an achlyoid spore dehiscence type (Scott 1961) which appears to be an ancestral character within the *Saprolegniaceae* (Leclerc, Guillot, and Deville 2000). Species of water molds in the genus *Aphanomyces* belong to an ecologically diverse group living as saprophytes or as parasites on fish, crayfish and plants.

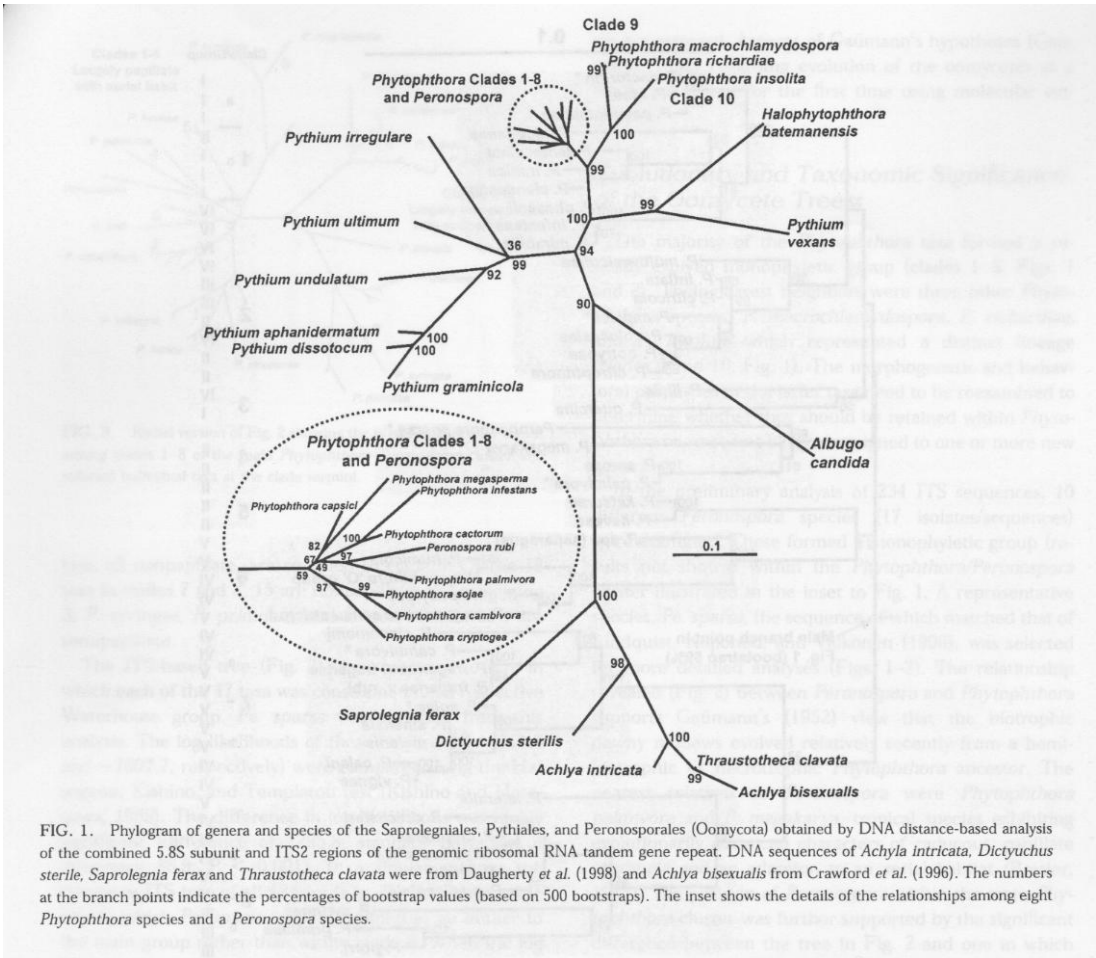

FIG. 1. Phylogram of genera and species of the Saprolegniales, Pythiales, and Peronosporales (Oomycota) obtained by DNA distance-based analysis of the combined 5.8S subunit and ITS2 regions of the genomic ribosomal RNA tandem gene repeat. DNA sequences of *Achyla intricata, Dictyuchus sterile, Saprolegnia ferax* and *Thraustotheca clavata* were from Daugherty *et al.* (1998) and *Achlya bisexualis* from Crawford *et al.* (1996). The numbers at the branch points indicate the percentages of bootstrap values (based on 500 bootstraps). The inset shows the details of the relationships among eight *Phytophthora* species and a *Peronospora* species.

Figure 2. Phylogram of genera and species of the Saprolegniales, Pythiales, and Peronosporales based on distance analysis conducted on rDNA sequences. Figure taken from Cooke et al. (2000).

Aphanomyces is a diploid, homothallic organism producing oospores and zoospores (Figure 3).

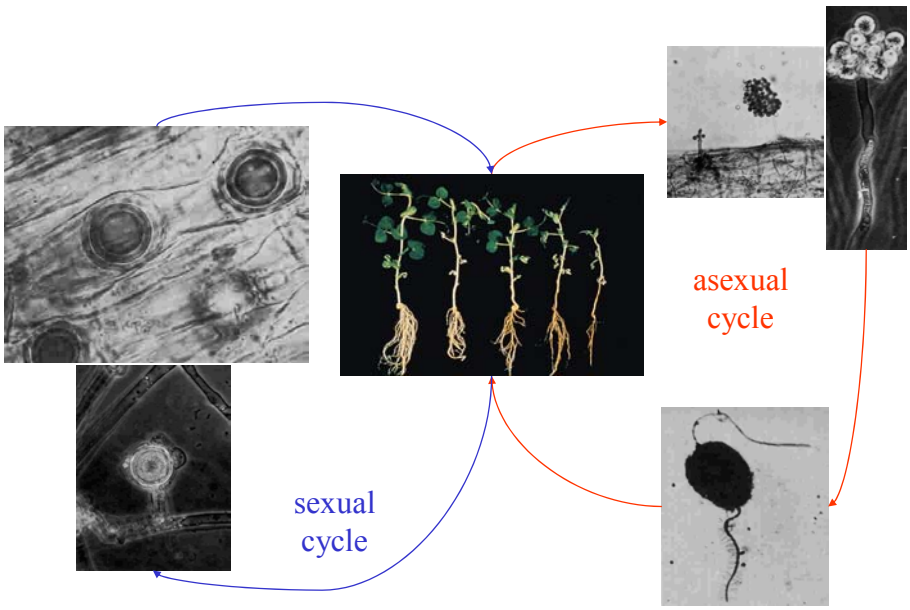

Figure 3. Simplified life cycle of the oomycete *Aphanomyces euteiches*.

The genus *Aphanomyces* includes 45 species and formae speciales currently distinguished (Table 1). Species in the genus *Aphanomyces* are described on single hosts (such as for example *A. iridis* on iris) or as having a broader host range (for example *A. euteiches* affecting several legume hosts) (Table 2).

Table 1. Species and formae speciales found in a search of the CABI Bioscience and CBS Database of Fungal Names. A total of 45 species and formae speciales are distinguished in this database.

Aphanomyces acinetophagus A.F. Bartsch & F.T. Wolf; Leptolegniaceae
Aphanomyces americanus; Leptolegniaceae
Aphanomyces amphigynus; Leptolegniaceae
Aphanomyces apophysii; Leptolegniaceae
Aphanomyces astaci Schikora (1906); Leptolegniaceae
Aphanomyces balboensis; Leptolegniaceae
Aphanomyces bosminae; Leptolegniaceae
Aphanomyces brassicae S.L. Singh & Pavgi (1977); Leptolegniaceae
Aphanomyces camptostylus Drechsler; Leptolegniaceae
Aphanomyces cladogamus Drechsler (1929); Leptolegniaceae
Aphanomyces cochlioides Drechsler (1929); Leptolegniaceae
Aphanomyces coniger H.E. Petersen; Leptolegniaceae
Aphanomyces daphniae Prowse (1954); Leptolegniaceae
Aphanomyces euteiches Drechsler (1925); Leptolegniaceae
Aphanomyces euteiches f.sp. euteiches; Leptolegniaceae
Aphanomyces euteiches f.sp. phaseoli W.F. Pfender & D.J. Hagedorn (1982); Leptolegniaceae
Aphanomyces euteiches f.sp. pisi W.F. Pfender & D.J. Hagedorn (1982); Leptolegniaceae
Aphanomyces exoparasiticus; Leptolegniaceae
Aphanomyces frigidophilus Kitanch. & Hatai (1997); Leptolegniaceae
Aphanomyces gordejevi; Leptolegniaceae
Aphanomyces helicoides Minden; Leptolegniaceae
Aphanomyces hydatinae; Leptolegniaceae
Aphanomyces invadans Willoughby, R.J. Roberts & Chinabut (1995); Leptolegniaceae
Aphanomyces iridis Ichit. & Tak. Kodama (1986); Leptolegniaceae
Aphanomyces irregularis W.W. Scott; Leptolegniaceae
Aphanomyces keratinophilus; Leptolegniaceae
Aphanomyces laevis de Bary (1860); Leptolegniaceae
Aphanomyces laevis f. keratinophilus; Leptolegniaceae
Aphanomyces laevis f. laevis; Leptolegniaceae
Aphanomyces laevis var. helicoides; Leptolegniaceae
Aphanomyces laevis var. laevis; Leptolegniaceae
Aphanomyces magnusii; Leptolegniaceae
Aphanomyces norvegicus Wille; Leptolegniaceae
Aphanomyces ovidestruens Gickelh.; Leptolegniaceae
Aphanomyces parasiticus Coker (1923); Leptolegniaceae
Aphanomyces patersonii; Leptolegniaceae
Aphanomyces phycophilus de Bary (1860); Leptolegniaceae
Aphanomyces pisci R.C. Srivast. (1979); Leptolegniaceae
Aphanomyces piscicida Hatai (1980); Leptolegniaceae
Aphanomyces polysporis; Leptolegniaceae
Aphanomyces raphani J.B. Kendr. (1927); Leptolegniaceae
Aphanomyces scaber de Bary; Leptolegniaceae
Aphanomyces sparrowii; Leptolegniaceae
Aphanomyces stellatus de Bary (1860); Leptolegniaceae
Aphanomyces volgensis; Leptolegniaceae

Table 2. Species of *Aphanomyces* described as plant pathogens including known hosts and names of diseases.

Species	Host	Disease
A. brassicae	Cualiflower (*B. oleracae*) Cabbage (*B. oleracae*) Kohl (*B. oleracae*) Not radish! Turnip (*B. rapa*) Mustard (*B. campestris*)	?
A. campostylus	oat	?
A. cladogamus	Tomato, spinach	Aphanomyces root rot
A. cochlioides	Sugar beet (*Beta vulgaris*) Table beet (*Beta vulgaris*) Isolated from: Lambsquarter (*Chenopodium album*) Spinach (*Spinacia oleracea*) New Zealand spinach (*Tetragonia tetragonioides*) Carpetweed (*Mollugo verticillata*) Bouncingbet (*Saponaria ocymoides*)	Black root rot Aphanomyces root rot
A. euteiches	Pea (*Pisum sativum*) Bean (*Phaseolus vulgaris*) Alfalfa (*Medicago sativa*) Red clover (*Trifolium pratense*) Subterranean clover (*Trifolium subterraneum*)	Common root rot, Aphanomyces root rot
A. iridis	iris	?
A. raphani	radish	Black root

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Impact of Aphanomyces Root Rot of Legumes in the Midwest

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SUMMARY

Aphanomyces euteiches is an important plant pathogen in the Midwestern US. Initially considered a root infecting pathogen of pea, *A. euteiches* has proven to be an important pathogen of bean, alfalfa and possibly other legume crops. Besides pathotypes based on host preference, races of *A. euteiches* are reported within the alfalfa and pea forms of the pathogen.

INTRODUCTION

Aphanomyces root rot is caused by the pathogen *Aphanomyces euteiches*. Pea, bean and alfalfa are the primary hosts of the pathogen, but other crops such as red clover are infected. As with most soilborne oomycetes, disease is most severe in flooded soil conditions and often associated with other root-rotting pathogens such as species of *Pythium* and *Phytophthora*. For example, the frequent failure of alfalfa varieties resistant to *P. medicaginis* lead to the discovery that *A. euteiches* is a significant pathogen of alfalfa. Symptoms and plant disruption caused by *A. euteiches* have similarities across hosts, but the perennial nature of alfalfa sets it apart from annual crops such as pea and bean. Regardless of crop, infection with *A. euteiches* can result in the death of seedlings, but more often results in stunted, chlorotic plants.

The pathogen is composed of several pathotypes based on host preference. RAPD markers have been found that characterize most isolates into groups related to pathotype based on host preference. Isolates within the alfalfa pathotype group also cluster into two and possibly more races based on reaction of a standard set of alfalfa populations. Populations of the pea pathotype grouping also differentially interact with a standard set of pea genotypes. It is critical to continue to investigate and characterize genetic variability for host preference within populations of *A. euteiches*. This knowledge can be used to tailor crop sequences and cover crops to reduce inoculum potential of the pathogen (Temp and Hagedorn, 1967; Wiliams-Woodward et al., 1997). The concept of races has been established within populations of the alfalfa and pea pathotypes. Although suspected, evidence is lacking for the existence of races within populations of the highly host specific bean pathotype.

MATERIALS & METHODS

Isolates of *A. euteiches* are commonly obtained from soil using different plant species as bait plants (Schmitthenner, 1964; Grau et al., 1991; Vincelli et al., 1991). The

plant species used to assay soils will have a dramatic affect on which pathotype of *A. euteiches* that is recovered. The semi selective medium, MBV agar (corn meal agar with 30 ppm metalaxyl, 5 ppm benomyl and 200 ppm vancomycin; Pfender et al 1984) can facilitate the recovery of isolates from soil and also field grown plants. However, it is frequently difficult to recover *A. euteiches* from field grown plants. Isolates are readily maintained on corn meal agar. Inoculation of plants should involve zoospores rather than mycelium or oospores. Zoospores are readily produced by one of several methods (Parke and Grau, 1992) and represent the most common form of natural inoculum. Plants are commonly inoculated at the seedling stage and the planting medium should be flooded for 5-7 days. Plants can be fertilized with Hoagland's solution or a similar fertilizer after, but not during the flooding phase due potential toxicity to zoospores. After 2 weeks of incubation seedlings can be rated using the following scheme: 1= no to very slight discoloration of roots; 2= slight necrosis of roots; 3= moderate necrosis of roots, slight chlorosis of cotyledons, and moderate stunting of stems; 4= extensive necrosis of roots, moderate to extensive necrosis hypocotyl and cotyledons, and severe stunting of stems; and 5= dead plant (Grau et al 1991). A pathotype designation is based on a rating of 4 or greater.

RESULTS & DISCUSSION

Studies have noted that isolates of *A. euteiches* tend to be most virulent on the crops from which they have been isolated (Malvick et al. 1998). In other words, isolates from alfalfa tend to cause more disease on alfalfa than on peas and vice versa. Exceptions do occur, and some isolates can cause severe disease on more than one crop (Malvick et al. 1998). The use of molecular tools has documented genotypic diversity among isolates of *A. euteiches* obtained from peas (Malvick and Percich 1998) and significant genotypic diversity among isolates obtained form different host species (Malvick et al. 1998). Isolates of the bean pathotype appear to represent the most host specialized group. The bean pathotype was isolated from bean, but not pea plants grown in naturally infested soils in Wisconsin (Table 1). Furthermore, the bean pathotype was more frequently recovered from bean stems than from bean roots. The pea pathotype was isolated from bean stems, but was recovered more frequently from bean roots. One isolate pathogenic to both bean and pea was isolated from bean roots. The vast majority of isolates recovered from pea stems and roots were pathogenic to pea. One isolate specific to alfalfa was recovered from pea roots. In contrast, 30 of 89 isolates recovered from bean roots were not pathogenic to bean and pea, but were pathogenic to alfalfa. Bean can be considered a "universal host" to *A. euteiches*.

Table 1. Frequency of pathotypes of *Aphanomyces euteiches* recovered from stems and roots of bean and pea

	No. of isolates of a specific pathotype per no. of isolates recovered			
Host part	Bean pathotype	Pea pathotype	Bean/Pea pathotype	Alfalfa pathotype
Bean stem	50/56	6/56	0/56	0/56
Bean root	38/89	21/89	1/89	30/89
Pea stem	0/95	95/95	0/95	0/95
Pea root	0/76	75/76	0/76	1/76

Breeding for resistance to *A. euteiches* has been most successful in alfalfa compared to pea and bean. WAPH-1 alfalfa germplasm was released by the Wisconsin Agricultural Experiment Station in 1989 (Grau 1992). This germplasm is resistant to Race 1 isolates of *Aphanomyces euteiches* . Resistance to Race 1 of *Aphanomyces* has been widely incorporated into commercial alfalfa varieties. Around 1990, isolates were recovered that were highly virulent to breeding lines with resistance to Race 1. Such isolates were recovered from soils collected in Wisconsin and eastern and southern states (Table 2). Similar isolates were also found in Iowa (Munkvold and Carlton, 1995). Such isolates were designated as Race 2 and represent a different form of the pathogen. Race 2 isolates of *A.* euteiches have now been found in Idaho, Maryland, Minnesota, Mississippi, North Carolina, Tennessee, Virginia, Iowa, Kentucky, and Wisconsin. Race 2 is therefore widely distributed in the United States. Alfalfa breeding programs are developing lines that are resistant to Race 2 of *A. eutieches*. Fortunately, these lines also appear to be highly resistant to Race 1 isolates as well. Race 2 resistant germplam would show the most pronounced effects where Race 2 populations of the pathogen predominate. However, even in regions where Race 1 populations predominate now, Race 2 may become more prevalent in the future if there is selection pressure by growing Race 1 resistant varieties). The variability that is present in populations of *A. euteiches* implies that breeders and growers must continue to be vigilant to meet the demands of controlling a changing pathogen population. This level of diversity in populations of *A. euteiches* provides challenges for breeding programs, because specific resistances may not be active against all strains of the pathogen.

Table 2. Reaction of four alfalfa populations to differentiate race 1 and race 2 isolates of *Aphanomyces euteiches.*

Alfalfa Populations	Resistance[a] Selection	Resistant Plants (%) Isolates			
		MF1	KY10	NC1	MPA22
Saranac	NR	1	8	0	0
Vernal	NR	9	6	1	2
WAPH-1	*R1*	*50*	*50*	*1*	*2*
WAPH-5	R1 & R2	60	63	50	50
LSD (p=0.05)		*10*	*18*	*11*	*6*

[a] Alfalfa populations were either not selected for resistance to *A. euteiches* (NR), or were selected for resistance to race 1 (R1) isolates or R1 and race 2 (R2) isolates. Isolates MF-1 and KY10 are race 1 and NC1 and MPA22 are race 2 isolates

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The impact of *Aphanomyces euteiches* from pea in France and disease control

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SUMMARY

Aphanomyces euteiches in France attacks mainly pea crops. Until now, other legumes don't seem affected by the disease. Only 4% of the cultivated area of pea is infested, but in some situations this disease can lead to a total destruction of the crop. The more efficient means of control currently available include prophylactic measures and substitution of pea by other legume crops.

INTRODUCTION

Since the 1990's, pea acreage has significantly decreased in France. In 2003, pea crop represented 400,000 ha for dried pea (360,000 ha spring pea, 40,000 ha winter pea) and 30,000 ha for processing pea (North Picardie, Brittany, South-West). One of the reasons of this decrease is the development of common root rot of pea caused by *Aphanomyces euteiches* Drechs. The disease appeared in France recently, causing important damages in the major pea-producing areas.

THE IMPACT OF *APHANOMYCES EUTEICHES* FROM PEA

Root rot severity in the world

This disease was described for the first time in the USA in the 20s (Jones & Dreschler, 1925), in Wisconsin where the pea crop was intensive since 1889, when the first factories were built. This new disease which mainly appears during wet and moderate growing seasons, was described in different parts of the USA where pea was cultivated (Papavizas & Ayers, 1974).

Later, this same disease was described in other parts of the world: North America, Australia, New-Zealand and Japan, on pea but also on other legume crops (Table 1).

In Europe, *A. euteiches* mainly is pathogen on pea (Oyarzun *et al.*, 1993, Bodker *et al*., 1993), but has also been isolated from root rot symptoms on faba bean in England (Salt & Delaney, 1986).

Root rot severity in France

In France, it was described for the first time by Labrousse (1933), but the main problems appeared in 1993 in the Parisian Basin (Didelot *et al.*, 1994), probably due to intensification of the pea crop from the 1980's and climatic conditions very favorable to the disease. The disease which initially appeared in the departments of Marne, Eure and

Eure et Loir, progressively spread in the different regions of dried pea production (Figure 1).

The infested acreage is estimated at 4% of the total area of dried pea. With some severe yield losses when climatic conditions are favourable (yield losses can reach 100% in some plots), this disease is considered as the greatest disease problem on spring dried pea in France. However, *A. euteiches* has never been reported on other legume crops in France. On winter pea and on Atlantic zones on spring pea, Ascochyta blight is the main problem.

DISEASE CONTROL IN FRANCE

Growers are organised to control disease in two main ways:

- prophylactic method: this method consisting in evaluating the inoculum potential of the soil before planting, is the more efficient (Didelot & Chaillet, 1995). This method established by the Plant Protection Service (from the agricultural ministry) consists in taking a sample of soil, to sow pea, to incubate in climatic chamber and to isolate the pathogen from the root symptoms (the cost is around 40 euros per test; around 2000 tests were requested by growers before planting in 2003). If *A. euteiches* is detected, growers take the decision to not sow pea in the plot. This method is efficient and permits the reduction of fields where disease is observed;

- substitution of pea crop by another legume crop such as faba bean or lupin. Until now, this way gives good results because faba bean is not infested by *A. euteiches* under field conditions in France. Maybe, it's a very precarious solution because there is a high probability to have in the future, isolates able to attack faba bean roots.

However there is no other way of control. A method using seed treatment by hyméxazol (Tachigaren, from Sumi Agro Factory) used against soil diseases of beet root has been tested, but the results show that the molecule is weakly efficient only with high doses.

In order to control Aphanomyces root rot, integrative disease management could be another solution. Several research programs have been developed in order to study three possible ways of control.

- cultural practices and rotations. This way is studied by pathologists from INRA in collaboration with growers institutes and extension services (UNIP, ITCF);
- genetic control. This way is much encouraged in France in public (INRA) and private (Group of Pea Breeders) research. From the American genotypes with partial resistance, crosses were realised, with the objective to breed resistant cultivars and to study the genetic basis of resistance. During this workshop, we will have many illustrations of this program.

The talks of Anne Moussart and Marie-Laure Pilet will illustrate the different approaches studied in research to contribute in the increase of knowledge potentially useful for the good control of the disease.

Table 1: Geographic distribution of *A. euteiches* in the different world regions (from Wicker'S PhD thesis, 2001)

Origine	References	Plant
North America		
⌦ ***USA*** : East and center states	(Jones & Drechsler, 1925)	**pea**, bean, alfafa, red
North West states	(Holub *et al*., 1991; Papavizas & Ayers, 1974)	trifolium
⌦ ***Canada*** : Ontario (1973)	(Basu *et al*., 1973)	**pea**
Manitoba (1979)	(Lamari & Bernier, 1985)	faba bean
Quebec (1989)	(Beghdadi *et al*., 1992)	**pea**, trifolium, alfafa
⌦ *Jamaica*	(Papavizas & Ayers, 1974)	**pea**
Australia (1955)	(Wade, 1955)	**pea**
Tasmania (1933)	(Allen *et al*., 1987; Othieno Abbo & Irwin, 1990)	bean, alfafa, trifolium
New Zealand (1977)	(Manning & Menzies, 1980)	**pea**
Japan (1974)	(Yokosawa *et al*., 1974)	**pea**
Europe		
Denmark (1927)	(Gram *et al*., 1928)	**pea**
France (1933)	(Labrousse, 1933)	**pea**
England and Wales (1951)	(Beaumont, 1951)	**pea**
	(Salt & Delaney, 1986)	faba bean
Sweden (1967)	(Olofsson, 1967)	**pea**
Norway	(Sundheim & Wiggen, 1972)	**pea**
Russia, Belorussia	(Kotova, 1979)	**pea**
Poland	(Furgal-Wegrzycka, 1984)	**pea**
Czech Republic	(Ondrej, 1988)	**pea**
Netherlands (1989)	(Oyarzun & van Loon, 1989)	**pea**

Fig 1: Regions of France infested by *A. euteiches* in 2000 (from predictive test of National laboratories of Plant Protection services

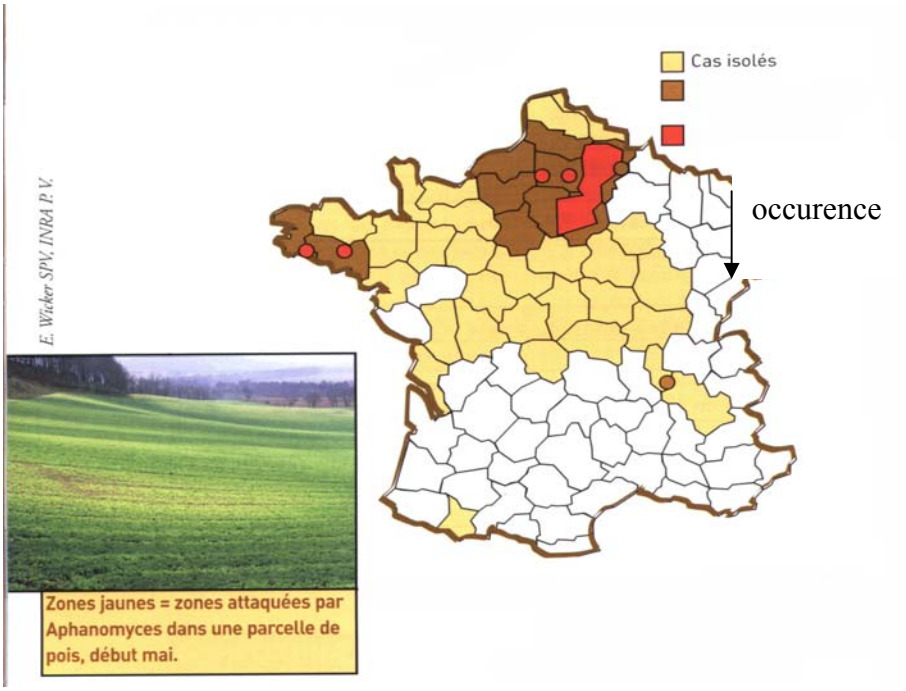
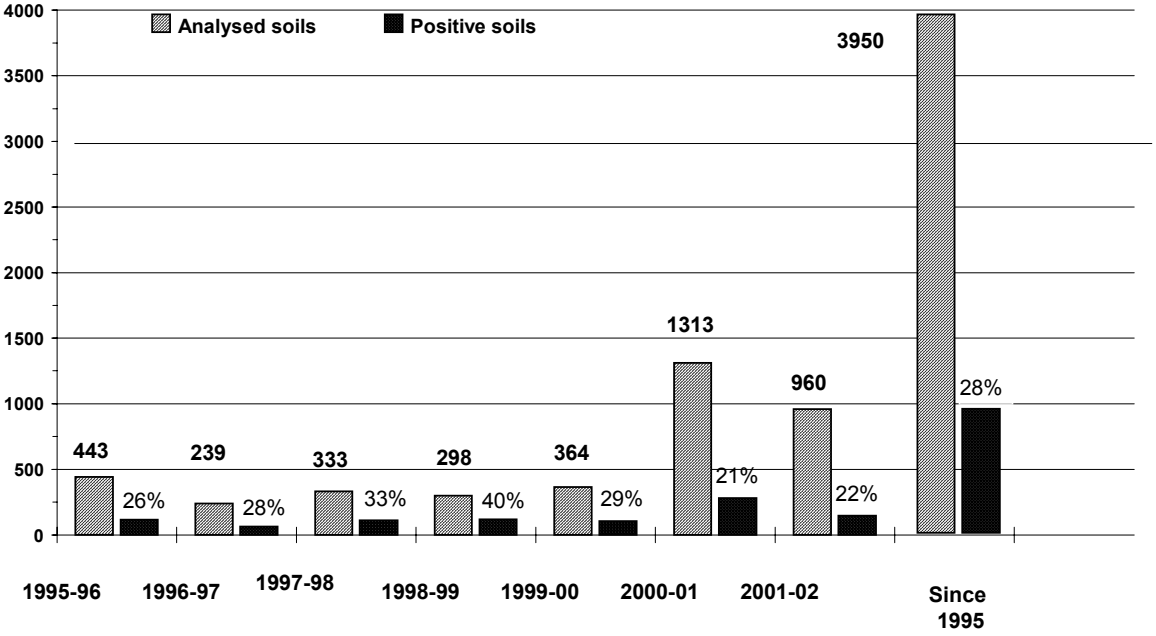


Fig 2 : Tests of detection of *A. euteiches* in soils
(from the Official Regional Laboratories of Plant Protection of Center of France and Champagne-Ardennes)



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Green manure crops and soil solarization effects on *Aphanomyces cochlioides*

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SUMMARY

A field experiment was conducted to determine effects of green manure crops and soil solarization on 1) suppression of Aphanomyces on sugar beet and 2) survival of *A. cochlioides* oospores. Following incorporation of green biomass into soil, sugar beet hypocotyls containing about 15,000 oospores were placed in nylon mesh bags (10 µm pores) and buried at three depths. Then, half of each plot was covered with polyethylene plastic for 7 weeks. A soil index value (0-100 scale) was determined after soil-incorporation of biomass before solarization. A pre-trial soil index value of 99 was reduced by all precrop treatments; oat resulted in the greatest reduction (79) and fallow soil, the least (96). During burial, hypocotyl tissue decomposed by about 60% across all treatments and only 1,675 oospores (11.2%) were alive. There was a significant and positive correlation between amount of hypocotyl tissue and number of living oospores buried in solarized soil at 8 and 23 cm, but not at 15 cm; there was no correlation for these factors in non-solarized soil at any depth. Overall, 1) green manure crops showed potential for reducing Aphanomyces damping-off and 2) oospore survival may be dependent on integrity of host tissue.

INTRODUCTION

Aphanomyces cochlioides causes seedling stand loss and chronic root rot of sugar beet (*Beta vulgaris*) when soil is warm and wet. Unusually wet summers in the last 10 years have favored increases in the prevalence and severity of Aphanomyces diseases on sugar beet in the Red River Valley of Minnesota and North Dakota and in west central Minnesota. In 1999, about 51% of acres planted to sugar beet in this region were estimated as infested with *A. cochlioides*. This pathogen produces oospores in infected roots, which presumably survive in soil for years, even when a sugar beet crop is not grown (Papavizas and Ayers, 1974). Little is known about factors affecting survival of oospores, but a visual technique was recently described to distinguish viable from dead oospores (Dyer and Windels, 2003).

Current control measures for Aphanomyces damping-off and root rot include early planting of partially resistant varieties, seed treatment with Tachigaren (hymexazol), water management (tiles or ditches to improve soil drainage, cultivation to dry soil), and weed control (*A. cochlioides* infects several common weed species, e.g., pigweed, lamb's-quarters, kochia). In fields with a high potential for disease, producers are advised to avoid planting sugar beet because if the season is wet and warm, control

options are inadequate and do not result in an economic return. Since disease control options are limited in effectiveness, additional strategies (e.g., green manure crops, soil solarization) are being explored. Green manure crops are reported to suppress several soilborne pathogens and pests on many crops, including suppression of Aphanomyces root rot by a green oat precrop (Williams-Woodward, *et al*., 1997; Windels, 1997). Soil solarization typically hastens decline of survival propagules of some soilborne fungi and other pests by generating high temperatures that directly kill propagules or weaken them so they are vulnerable to parasitism by other organisms (Katan, 1987). Solarization is reported to be effective in temperate regions when combined with green manure crops, reduced dosages of chemicals, or biological control organisms (Katan, 1987; Ramierz-Villapudua and Munnecke, 1988).

The objective of this research was to determine the effect of several green manure crops and soil solarization on 1) suppression of Aphanomyces root rot on sugar beet and 2) survival of oospores of *A. cochlioides*. This report includes a portion of a 2002 field trial in a project conducted in 2001-2004.

MATERIALS AND METHODS

Precrop and solarization treatments. The trial was initiated on May 24, 2002 in a commercial field near Crookston, Minnesota that was naturally infested with *A. cochlioides*. Several crops were sown including buckwheat (*Fagopyrum esculentum* 'Koto'), oilseed radish (*Raphanus sativus* subsp. *oleiferus* 'Colonel'), sorghum sudan grass (*Sorghum bicolor* 'Green Grace Supreme'), oat (*Avena sativus* 'Dane') and wheat (*Triticum aestivum* '2375'). Wheat is commonly grown the season before sugar beet and the other crops are reported to suppress various soilborne fungal diseases on field crops. The control was fallow soil. Each plot measured 12 x 9 m and was arranged in a randomized block design with four replicates.

At planting, soil cores (six, 6-cm diameter) were collected to a depth of 15 cm and combined per plot. Soil samples were evaluated by a sugar beet seedling assay in the greenhouse and Aphanomyces soil index values were determined (Windels and Nabben-Schindler, 1996). Aphanomyces soil index values are based on a 0 to 100 scale where 0=healthy and 100=all sugarbeet seedlings died during the 4-week assay.

On July 16 (7 ½ weeks after planting), all green manure crops were mowed and the residue was disked and rototilled into soil to a 10-cm depth (soil was too dry and compacted to incorporate residue deeper). Quantities of buckwheat, oilseed radish, sorghum sudan grass, oat, and wheat averaged 13, 38, 30, 27, and 22 mT fresh weight/ha, respectively. Fallow control plots also were disked and rototilled. Each main plot (green manure crops and fallow) then was split into subplots (one for solarization and the other not solarized). Soil samples were collected in each subplot and were indexed for Aphanomyces root rot in the greenhouse. Thermocouples were buried at 8, 15, and 23 cm in subplots of one replicate and soil temperatures were recorded on a Watchdog data logger (Spectrum Technologies, Plainfield, IL) every 15 minutes. Solarized plots were covered with a clear, horticultural grade polyethylene plastic (3 mil) on July17; edges of tarps were buried in furrows. After tarps were applied, 1.25 cm precipitation fell in July and 17.3cm in August.

Oospore survival. Oospores of *A. cochlioides* also were buried in soil to observe the effect of green manure crops, with and without soil solarization, on survival. Oospores were produced by placing excised, 2-cm length segments of 2-week-old sugar beet hypocotyls in sterile water, which were inoculated with mycelial disks of *A. cochlioides* and incubated in the dark at 23 °C for 7 weeks. Oospores averaged 15,000 per hypocotyl. One hypocotyl segment was placed in the bottom of a nylon monofilament mesh fabric (< 10µ pores) bag (2.5 x 2.5 cm), which was closed with string and placed in a pan of water to prevent drying. All hypocotyls had been microscopically examined to ensure presence of oospores. Bags with oospores were buried at 8, 15, and 23 cm depths in each green manure crop and fallow subplots designated to be solarized or not solarized.

Tarps were removed after 7 weeks. Oospores were removed, placed in plastic bags, moistened with water, and stored in a refrigerator until examined. Each bag was carefully opened along the outside seams. Hypocotyls were removed and microscopically inspected to determine the amount of tissue. Relative amounts of hypocotyl tissue were assessed on a 0-5 scale: 0 = no tissue present, 1 = 1-20% of original tissue present (or only vascular tissue remaining), 2 = 21-40%, 3 = 41-60%, 4 = 61-80%, and 5 = 81-100% of original tissue intact. After each hypocotyl had been assessed, it was placed in a 2 ml Wheaton tissue grinder; 1 ml of distilled water was added; and the plunger was depressed fifteen times. Contents were transferred to a 1.5 ml tube and centrifuged 10 minutes at 10,000 rpm. A 750 µl aliquot of supernatant (not containing oospores) was decanted and the remaining 250 µl (containing oospores and macerated hypocotyl tissue) was vortex-mixed for a few seconds. Aliquots of the resulting oospore suspension were placed on a Speirs-Levy eosinophil counting slide to determine total number of oospores (living plus dead) and number of living oospores (Fig. 1A and B).

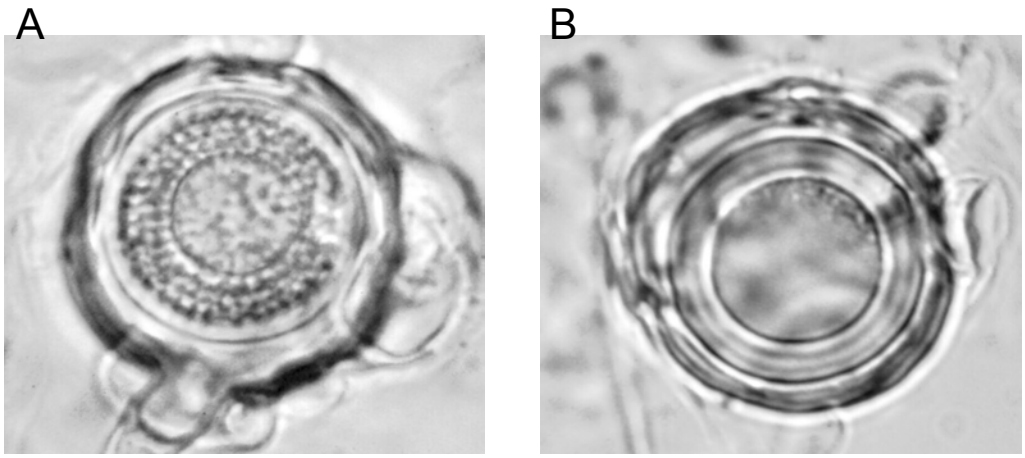

Fig. 1. Oospores of *Aphanomyces cochlioides* with: **A)** a densely organized uniform granular appearance typical of living oospore compared to **B)** a loosely organized nonuniform granular appearance of a dead oospore.

Data analysis. Data for relative amounts of sugar beet hypocotyl tissue, number of total oospores (living and dead), and number of living oospores were subjected to

appropriate transformations (if needed) and Analysis of Variance. If significant (P = 0.05), means were separated by Least Significant Difference (LSD). Correlations were calculated for relative amounts of hypocotyl tissue and living oospores.

RESULTS AND DISCUSSION

Greenhouse assay for Aphanomyces soil index values. Before green manure crops were sown in 2002, the average soil index value was 99 (data not shown). After incorporation of green manure crops (and before solarization), soil index values were reduced by all precrop treatments by varying amounts (data not shown). Oat resulted in the greatest reduction of the soil index value (79); buckwheat and sorghum sudan grass had slight and equal reductions (90), and oilseed radish and wheat were intermediate (86 and 83, respectively). Soil index values in fallow plots were reduced slightly (96). In 2003, a sugar beet crop was sown in this trial to determine effects on disease and crop yield. Previous studies have shown that a green manure oat crop results in excellent suppression of Aphanomyces root rot of sugar beet in naturally infested soil in the greenhouse but does not perform consistently in the field (Windels *et al*., 1993). Disease suppression by green manure crops may be short-term and not carry over the winter to benefit a subsequent sugar beet crop. A green oat precrop, however, reduced Aphanomyces root rot of peas caused by *A. euteiches* (Williams-Woodward *et al*., 1997).

Soil temperatures. Solarization resulted in maximum soil temperatures of 43, 37, and 32 °C at 8-, 15-, and 23-cm depths, respectively (data not shown). Non-solarized soil attained maximum temperatures of 33, 30, and 27 °C at 8-, 15-, and 23-cm depths, respectively (data not shown). The highest ambient temperature during solarization was 32 °C on August 2, 2002. There are no reports documenting the effects of solarization on *A. cochlioides* but Dyer (2002) has shown that 90% of oospores die when exposed to 40 °C for 72 h or 50 °C for 4 h. He further determined that effects of such "lethal" temperatures are cumulative when temperatures fluctuate. Temperature data collected in the field in 2002 still needs to be analyzed for duration and frequency of occurrences at "lethal" temperatures. If soil temperatures achieved in the field during solarization are insufficient to kill oospores of *A. cochlioides*, they may predispose them to microbial decomposition (Katan, 1987).

Oospore survival. Examination of sugar beet hypocotyls and oospores removed from soil immediately after burial revealed various stages of tissue decomposition. Some hypocotyls were fairly intact and the cortex contained abundant oospores. In other cases, the cortex was severely decomposed and only vascular tissue, which contained a few oospores, remained. Occasionally, no hypocotyl tissue or oospores remained or only a few oospores were attached to the interior of the mesh bag. *A. cochlioides* oospores average 21 $\pm$ 5 µ in diameter and would be too large to pass through 10µ pores of the nylon mesh bags. Oospores rarely survived or were not found in mesh bags containing severely decomposed sugar beet hypocotyls, indicating that all or most had already died and decomposed.

Table 1. Survival of *Aphanomyces cochlioides* oospores within sugar beet hypocotyls that were buried in the field at three depths within 1 day after green manure crops had been incorporated into soil. Plots then were solarized (July 17 - September 4, 7 weeks); controls included non-solarized plots of each green manure precrop and fallow plots (solarized and non-solarized). Immediately after solarization, hypocotyls were retrieved and oospores were microscopically assessed for viability

	Number of oospores[y]	
Treatment	Total (living + dead)	Living
Precrop		
Buckwheat	8,040	1,470
Wheat	7,580	1,900
Sorghum sudan grass	7,430	1,770
Oat	7,040	1,660
Fallow	6,280	1,460
Oilseed radish	5,950	1,790
Mean	7,050	1,675
LSD ($P \leq 0.05$)[z]	NS	NS
Soil treatment		
Solarized	7,180	2,230
Non-solarized	6,920	1,117
Mean	7,050	1,675
LSD ($P \leq 0.05$)[z]	NS	809
Depth (cm)		
8	6,670	1,770
15	6,920	1,420
23	7,750	1,840
Mean	7,050	1,675
LSD ($P \leq 0.05$)[z]	NS	NS

[y] Number of living oospores per 2 cm length of hypocotyl before burial in soil was 15,000.

[z] LSD=Least Significant Difference; if significant, LSD value provided for mean separations; NS = not significant.

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The relative amount of hypocotyl tissue remaining after burial for 7 weeks averaged 2.6 and was unaffected by green manure crops or the fallow control, solarized and non-solarized treatments, and depth of burial (data not shown). A rating of 2 = 21 to 40% of original tissue buried in soil was intact and 3 = 41 to 60% was intact.

Viability assessments of oospores are summarized in Table 1. There were no significant interactions between main treatments, so data are presented only for main treatments. Of an average of 7,050 oospores (living plus dead) remaining in hypocotyl tissue buried in soil, 1,675 were alive. An average of 15,000 viable oospores originally were present in hypocotyls before burial, so survival after burial in soil for nearly 2 months was only about 11%. Numbers of living oospores were not significantly affected by green manure crop and fallow treatments (Table 1). Total numbers of oospores were

the same in solarized and non-solarized soils, but the number of living oospores was significantly higher in solarized soil. Oospore viability was not significantly affected by depth of burial.

There was a significant and positive correlation between amount of hypocotyl tissue and number of living oospores buried in solarized soil at 8 and 23 cm, but not at 15 cm (Fig. 1A). There was no correlation for these factors in non-solarized soil at any depth (Fig. 1B).

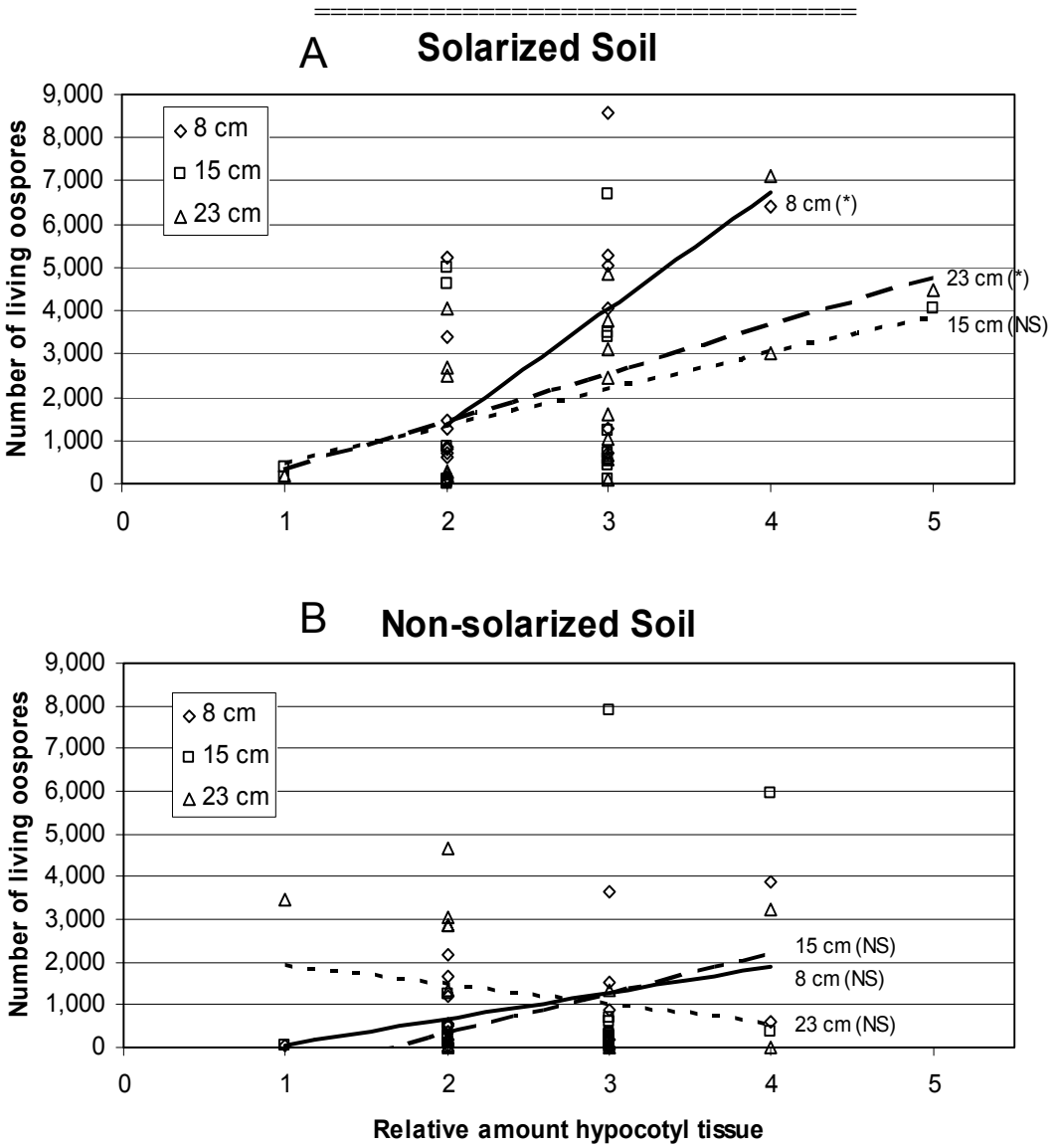

Fig. 2. Relationship between relative amount of sugar beet hypocotyl tissue and number of living oospores of *Aphanomyces cochlioides* (hypocotyl originally contained about 15,000 oospores/2-cm segment) after burial in field plots at 8, 15 and 23, cm that then were **A)** solarized and **B)** not solarized for 7 weeks. Relative amount of hypocotyl tissue based on a 0-5 scale; 0=no tissue present and 5=81-100% of tissue originally buried was intact. NS=not significant; *=significant $P \leq 0.05$.

Solarization may have resulted in less microbial activity and thereby, retarded decomposition of hypocotyl tissue and oospores compared to non-solarized plots. A similar study by the authors in 2001, showed a direct relationship between oospore survival and amount of hypocotyl tissue, which suggested dependency on host tissue for survival. Weather conditions in 2002 may have been more favorable for microbial activity than in 2001 and resulted in more rapid decomposition of hypocotyls.

Observation of a rapid decline in oospore survival when *A. cochlioides*-infected hypocotyls decompose in soil has not been previously reported, although Boosalis and Scharen (1959) found oospores of *A. euteiches* associated with diseased plant debris extracted from soil. Sugar beet hypocotyl tissue, however, is immature and delicate and decomposes readily. Oospores of *A. cochlioides* may survive longer in mature sugar beet roots, which have secondary thickenings and decompose slowly in soil. Pfender and Hagedorn (1983) reported a substantial loss of inoculum of *A. euteiches* of nearly 50% within 1 year after growing pea (based on a most probably number assay). Survival of only a few oospores in a small volume of soil, however, can be deceptive since a single oospore of *A. cochlioides* produces up to 300 zoospores of primary inoculum. Consequently, a low concentration of oospore inoculum in soil can result in considerable disease in a growing season that has prolonged warm and wet conditions.

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An Industry Perspective On Aphanomyces Root Rot On Fresh (Processed) Peas

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SUMMARY

Aphanomyces root rot in peas affect growers and processors by reducing volumes and quality. Growers generally receive a lower price due to over-mature peas in payment grade samples. Quality to consumers is also lowered due to maturity issues. Disease management is currently by field avoidance either by soil testing or crop rotation history. Traditionally developed (no biotech) varieties are currently needed that have tolerance/resistance. Biotechnology may be the future for management of Aphanomyces, but consumers must approve of the technology first.

DISCUSSION

Aphanomyces in peas for processing is an ongoing concern that needs to be managed for maximum returns to both processor and grower.

Production of processed peas requires varieties that have been tested and proven for traits that are desired. Growers demand varieties with high yields and low inputs. Processors also want varieties with high yields. Varieties should also process with high recovery of peas that are delivered to the processing plant. High quality attributes are also important to processors.

Root rot diseases of peas (especially Aphanomyces) affect the desired traits of a variety. Bottom line dollar impact and production volumes are most affected. For example, suppose a hypothetical one hundred-acre pea field in the Columbia Basin of Washington State yielded 3.5 tons per acre. A potential price might be $150.00 per ton, netting a return of $525.00 per acre to the grower. Should this field have a 30 percent yield loss due to Aphanomyces, yield would then be 2.45 tons per acre. This is a $157.50 per acre loss of income to the grower. Loss could be greater if Aphanomyces impacts maturity at time of harvest.

Production volumes are also reduced as a result of Aphanomyces. Additional acres are needed to cover volume shortfall. This increases cost to the processor due to additional seed purchases, pest control, harvest machinery costs, or purchases of raw/finished product from other processors.

Quality of finished processed peas may also be affected due to over-maturity or uneven maturity. Limits are placed on grade standards. Selling grade “B” peas versus grade “A” can be difficult.

Contracting acres free from Aphanomyces is becoming difficult in the Columbia Basin of Washington State due to other crops, rotations, and land leasing issues.

Needs of the processor include varieties from traditional breeding techniques that have tolerance/resistance to Aphanomyces. A chemical, either seed treatment or broadcast soil treatment would be helpful. Biotechnology offers possibilities for solutions, but consumer acceptance of biotech peas would be necessary. Cost of biotech peas would also be a concern.

Impact and Characteristics of *Aphanomyces euteiches* Associated with Alfalfa in the Midwestern U.S.

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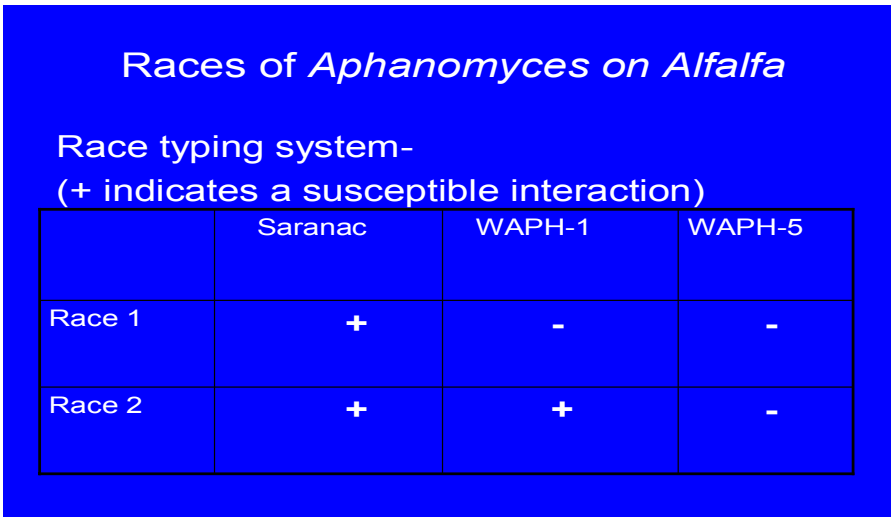
Aphanomyces euteiches Drechs. was first associated with infection of alfalfa in 1927 (3). Isolates of *A. euteiches* that infected alfalfa were described more completely 1964, but it was not until the 1980's that Aphanomyces root rot was reported to be an important and widespread disease of alfalfa (1, 7). Aphanomyces root rot of alfalfa has been reported in North America from Quebec, Canada and several U.S states including Iowa, Kentucky, New York, Virginia, and Wisconsin. This disease is destructive to seedling stands of alfalfa in slowly-drained soils, and also can cause chronic stress to mature plants (8). *Aphanomyces* has also been associated with root heaving and associated winter damage (10). It can occur in combination with Phytophthora root rot to result in a severe wet-soil root rot complex. Under natural conditions in Kentucky, alfalfa cultivars rated resistant or highly resistant to Aphanomyces root rot provided significantly increased seedling health and yield compared to susceptible varieties (9). Chemical controls for Aphanomyces root rot are not available for legumes, and this disease is best managed with resistant cultivars and by avoiding poorly drained, highly infested fields. Breeding for disease resistance has been much more successful for alfalfa than pea.

Variation in virulence among isolates of *A. euteiches* from alfalfa has been reported for isolates from several states including Maryland, Minnesota, North Carolina, Virginia, and Wisconsin (2). Most of the isolates reported, which are now designated race 1 (R1), were highly virulent on the susceptible alfalfa cv. Saranac and expressed low virulence on WAPH-1, an alfalfa population with R1 resistance similar to most resistant commercial cultivars. Isolates have been reported from several states that are virulent on Saranac and WAPH-1 and are now designated race 2 (R2). Cultivars with resistance to R1 and R2 of *A. euteiches* had significantly greater yields than varieties with no resistance to Aphanomyces root rot or resistance only to R1 at a Wisconsin location infested with *A. euteiches* R2 (6). Until recently it was unknown whether the R2 isolates represent a widespread risk to resistant commercial cultivars, which were developed using R1 isolates for screening.



The research to address this question has been focused on the Midwestern U.S. Aphanomyces root rot of alfalfa was known to occur in multiple locations in Wisconsin, Kentucky, and Iowa. However, little was known about the distribution, characteristics, and impact of this disease in Illinois. In Illinois, *Aphanomyces* was only known to occur in alfalfa fields in the northwest corner of the state near the Wisconsin border, and this was poorly understood and documented. The occurrence, characteristics, and frequencies of races 1 and 2 of *Aphanomyces* were first studied in Wisconsin, Minnesota, and Kentucky. Because *Aphanomyces* had not been studied in Illinois, the impact of this disease, the occurrence and distribution of the different races, and the potential value of using alfalfa cultivars with resistance to *Aphanomyces* in Illinois was not known.

Results from these studies have suggested Aphanomyces root rot is a widespread and significant disease of alfalfa in the Midwestern U.S.A. In Iowa, *A .euteiches* was isolated from about 35% of the soil samples tested from across the state (5). Information on occurrence and distribution of R1 and R2 in Iowa have not been published. Another study focused on Wisconsin, Kentucky, and Minnesota (4). The distribution, frequency, and pathogenic and genotypic characteristics of race 1 (R1) and race 2 (R2) isolates were studied in 13 fields in Wisconsin (405 isolates), seven fields in Minnesota (4 isolates), and one field in Kentucky (48 isolates). *A. euteiches* was successfully isolated from the soil of 17 of the 21 fields. Pathogenicity and race phenotype of isolates were characterized on Saranac (susceptible to R1 and R2 isolates) and WAPH-1 (resistant to R1 and susceptible to R2 isolates) alfalfa populations. In one Wisconsin field with no recent history of alfalfa production 51% of the isolates were R2, and 43% of all isolates were R2 from fields with a history of alfalfa production. In a location that was planted continuously to pea for 30 years, 27% of the isolates were R2. Three R1 and three R2 isolates were subjected to RAPD analysis with eight primers, however, none of the PCR-generated amplicons were uniquely associated with race phenotype. Evaluation of eight commercial alfalfa cultivars for resistance to two R1 and two R2 isolates demonstrated that most are susceptible to R2 isolates, and only those selected for R2 resistance were resistant to R2 isolates (4).

More recent work suggests *A. euteiches* is also very widespread in Illinois (D. Malvick, unpublished data). Soil samples were collected from 35 alfalfa fields in 19 counties concentrated in the primary alfalfa production areas of Illinois. *Aphanomyces euteiches* (311 isolates) was isolated from 85% of the soil samples, including all counties, using cultivar Saranac as a baiting host. Race phenotype of 140 isolates was characterized on 'Saranac' and `WAPH-1'. Approximately 60% of the isolates were R1 and 40% were R2. Both races were isolated from 67% of the counties, whereas only R1 or R2 was isolated from 17% of the counties. These results indicate that *Aphanomyces euteiches* is a common pathogen in Illinois.

The results from several studies suggest that R2 isolates represent a widespread risk in the midwestern U.S. to alfalfa cultivars having resistance only to R1 isolates in fields with varied cropping histories. Furthermore, Aphanomyces root rot may be best managed in many areas with cultivars having resistance to both R1 and R2.

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The Impact of *Aphanomyces* Root Rot in the Pacific Northwest

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Processed (canned and frozen) green peas, in the Blue Mountain region of Northeastern Oregon and Southeastern Washington, have been grown in rotation with winter wheat for over 60 years. During the boom years of the 1950's and 1960's over 80,000 acres of peas were processed in plants from Pendleton, Oregon to Dayton, Washington.

Acreage gradually declined until the acreage, during the 1980's and 1990's, stabilized, by the late 1990's, at approximately 35,000 acres grown for freezing and 3,000 to 5,000 acres grown for canning. A major freezing plant in Walla Walla, WA, that normally processed 16-18,000 acres of peas, closed after the 2000 season. Factors contributing to this acreage decline include:

1) Declining or slightly stable per capita consumption in the United States;
2) Competition from other countries;
3) Variable climatic conditions that result in yield swings from 500 to 6,000 pounds of peas per acre (the area's long-term yield is 1.25 to 1.50 tons/acre);
4) Buildup of soil borne fungal diseases, such as *Pythium, Rhizoctonia, Fusarium* root rot, Race 2 *Fusarium* wilt and *Aphanomyces,* that negatively impact yield and product quality.

Virus diseases transmitted by the green pea aphid, such as enation, and in 2002, bean leaf roll, can occasionally be serious, especially after mild winters. After the first cutting of alfalfa (mid May) west of the pea production, aphid flights move into the green pea crop.

Processors have increased irrigated (center pivot) acreage in the Columbia Basin region. Grown on light sandy and silt loam soils, the combination of irrigation and soil fertility can result in excessive vine growth. *Sclerontia* or white mold is increasingly becoming a problem. Processing companies suspect that *Aphanomyces* is on the increase there.

Over 90 per cent of the peas in the Blue Mountain region are dry land and grown in silt loam soils. Nitrogen fertilization in the winter wheat crop has caused a decline in soil pH. Many fields have a pH of less than 5.5. Average annual precipitation varies from 16 inches to over 25 inches. Pea planting begins in March, in fields at 800 to 1,000 feet elevation. The majority of the fields are fall plowed and spring-toothed in the spring to incorporate herbicides and prepare the seedbed. Most growers broadcast 100 pounds of ammonium sulfate fertilizer on the wheat straw prior to plowing. Often fields are worked too wet, increasing soil compaction and soil borne disease incidence. Field conditions can be very wet and cold after seeding and it is not unusual for early seeded peas to take three weeks to emerge. Planting continues until the higher elevation (2,500-3,000 feet) fields are seeded in early to mid May. With elevation differences and the use of 15 to 20 varieties the usual harvest period is from early June to late July. Occasionally harvest will start in late May and extend to early August.

Growing conditions are highly variable with much of the acreage receiving little to no precipitation after early June. Fields at the higher elevations may receive precipitation later in June and early July. The maximum daytime temperature at harvest many times exceeds 100 degrees F. with a relative humidity of less than 15 percent.

I believe the impact of *Aphanomyces* on green pea production in the Blue Mountain and Columbia Basin regions is not fully understood due to:
1) The incidence and interaction of soil borne diseases common to the regions;
2) Extremely variable field and climatic conditions and their impacts on disease expression and incidence;
3) The influence of supplemental irrigation;
4) The impact of reduced tillage on the soil biosphere.

I recommend a systematic sampling of representative fields to determine the incidence and levels of soil borne diseases. I believe this is a basic research need that will lead to a better understanding of the interaction of the various soil borne diseases and the impact of *Aphanomyces* on the regions green pea industry.

Section II: Epidemiology, population genetics, and host-parasite interactions

Pathology research programs in France to control Aphanomyces root rot

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SUMMARY

Aphanomyces root rot is the most damaging root disease of pea in France. Pathology research programs have been established since 1997 in order to get a better knowledge of the disease and to develop integrative disease management programs.

INTRODUCTION

In France, common root rot caused by *Aphanomyces euteiches* Drechs. has caused major losses in pea crops since 1993 (Tivoli and Moussart, 2003). Initially reported in the Parisian Basin, the disease has spread into all the regions of pea production and is now considered as the most damaging root disease of pea in France. In order to control Aphanomyces root rot, several pathology research programs have been developed at INRA since 1997 in collaboration with the legume growers union (UNIP). The first objective is to have a better knowledge of the disease, in order to study different ways of control (genetics, rotations and cropping practices). Three main topics are investigated: pathogen variability, resistance and epidemiology.

PATHOLOGY RESEARCH PROGRAMS

Pathogen variability

For a better understanding of the origin of the epidemic and to support research efforts for genetic and cultural control, the pathogenic and molecular variability of the French populations *of A. euteiches* was investigated (Wicker, 2001).

The study of host specificity showed that French isolates were preferentially pathogenic on pea but could infect other legume species (vetch, alfalfa, faba bean and snap bean) (Wicker *et al*., 2001a). To investigate the variability in virulence on pea, a set of six differential genotypes was established (Wicker *et al*., 2003). A main virulence type was found (Wicker *et al*, 2001b). The French isolates appeared to be more aggressive than foreign ones and were virulent on lines identified as partially resistant to the foreign

isolates. A high genotypic diversity was revealed using AFLP markers but no structuration of the French population was found.

Resistance

A close collaboration has been developed between pathologists, geneticists from INRA (M.L. Pilet-Nayel and A. Baranger) and private breeders from GSP (group of seven pea breeders).

The pathology group has been involved in developing methodologies for screening for pea resistance to Aphanomyces. A standardised screening test in controlled conditions has been developed (Moussart *et al*., 2001). This test, simple and reproducible, has been validated by breeders. A positive correlation has been established between screening in the field and screening in controlled conditions. The test is also used for many other studies, especially in pathology.

Since 2002, expression of partial resistance has been assessed. The main objective is to find the components of partial resistance that reduce the rate of epidemic development, using genotypes expressing different resistance levels. Experiments are conducted in field trials and in controlled conditions. The first results have led us to underline the different phases of the epidemic and to identify two resistant components.

Several research programs are developed at INRA on the model Legume *Medicago truncatula*. In pathology, resistance to Aphanomyces in *M. truncatula* has been studied for two years. A first screening in the French core collection of *M. truncatula* (J.M. Prosperi, INRA Montpellier) allowed to discover a large variation of susceptibility with high levels of partial resistance. A differential set of *M. truncatula* ecotypes will be established in order to compare resistance to Aphanomyces between pea and the model Legume.

Epidemiology

Epidemiology studies are necessary to get a better knowledge of the disease in order to develop ways of control such as cropping practices or rotations.

The first study conducted at INRA was about spatial distribution of the disease in one field. The main objective was to have informations about inoculum distribution in relation with topography. This study was also carried out to have spatial mapping of the root rot potential in one field, to establish field experiments in areas characterized by their level of infestation. In order to study horizontal distribution of the pathogen (hot plots), two methods of mapping were developed : a mapping by measuring inoculum potential of soil and a mapping by evaluating disease severity on plants. A positive relationship was found between the two methods. Vertical distribution of the pathogen (in depth) was investigated by measuring inoculum potential in layers of soil until 60 centimeters depth. The vertical level of infestation is correlated with the horizontal level of infestation but the fungus was always find until 60 centimeters in depth at least. No relationship was establish between inoculum distribution and topography.

Impact of rotations on the incidence of Aphanomyces root root has been investigated since 2001. Antifungal activities of different green manures such as cruciferous are studied in the field and in controlled conditions. The strategy is i)to screen several varieties of cruciferous species for their ability to reduce the disease and ii)to study effect of cropping practices and climatic conditions on the effectiveness of these

green manures for suppressing root rot. Such a study requires a close collaboration between pathologists, soil biologists, microbiologists and chimists. Another study concerns the consequences of cultivating other legume crops in infested areas. According to results of host specificity study (Wicker *et al.,* 2001a), *A. euteiches* is not specialised on pea. However, the pathogen has never been reported on any other crops in France. So, the relationship between cropping frequency of other legumes and occurrence of Aphanomyces root rot is investigated in order to evaluate the possibility of infection of other legumes.

Since this year, we have been also interested in studying the influence of climatic factors on epidemic development and the relationship between plant stage and disease severity.

CONCLUSION

Pathology research programs are necessary for a better understanding of Aphanomyces root rot aiming at identifying breeding and cultural solutions. In order to have a good integrated control of the disease, a close collaboration is necessary between pathologists, geneticists, private breeders and Legume growers union. An integrative disease management is probably the solution to control Aphanomyces root rot and would most probably allow to increase pea production in France

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Host-pathogen interactions in the phytopathogenic Aphanomyces.

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SUMMARY

Bulk assay and gel activity assays were used to characterize proteases secreted by *Aphanomyces cochlioides* and *A. euteiches* both in culture and in infected seedlings. Using bulk assays, inhibitors of trypsin-like enzymes were capable of reducing sample protease activity, whereas inhibitors for other protease classes had no effect. Non-denaturing sodium dodecylsulfate polyacrylamide gel electrophoresis separated protease isozymes secreted by *A. cochlioides* into 7-8 resolvable bands whereas those secreted by *A. euteiches* were resolved into 6 bands. Incorporation of trypsin-class inhibitors either into the electrophoresis gels or into the activity staining buffer resulted in a decrease of activity for the fastest migrating isozymes. The involvement of protease activity in the infection of plants by Aphanomyces and the implications for disease control are discussed.

INTRODUCTION

Phytopathogenic fungi and oomycetes that induce root rot disease have received intense study with regards to the production of cell wall degrading enzymes (CWDEs; Walton, 1994). This is due to the fact that, for many host-pathogen combinations, the infiltration of host tissue with secreted enzymes of the corresponding pathogen will induce tissue maceration similar to that characterizing the disease. Enzyme activities that fall under the category of CWDEs include cellulase, pectinase (broadly comprised of polygalacturonase, pectin methylesterase, and pectin and pectate lyase), xylanase, and protease (Walton, 1994).

Although CWDEs produced by root rot pathogens have been implicated in pathogen virulence, proof of their involvement by the application of defined inhibitors or pathogen mutants has been lacking. Defined mutants have been used, however, to investigate the role of CWDEs in the virulence of several haploid phytopathogenic fungi that incite stem or leaf diseases. Examples include the determination of pectin lyase activity as a virulence factor in *Nectria hematococca* (Rogers et al., 2000) on pea and the discovery of endopolygalacturonase as a virulence factor in the infection of tomato by *Botrytis cinerea*(Ten Have et al., 1998).

One could envision protease activity as a virulence factor in phytopathogens, participating in both the weakening of host cell walls as well as in the inactivation of pathogenesis-related (PR) proteins induced in the plant. Although the involvement of

proteases in the virulence of mammalian fungal pathogens has been known for decades, few clear examples of protease involvement in the induction of plant disease exist among the plant pathogenic microbes. Limited examples include the production of protease by *Fusarium eumartii* (Olivieri et al, 2002) and *Phytophthora infestans* (Paris and Lamattina, 1999).

The oomycete genus Aphanomyces is remarkable for its broad host range as a pathogen. The host range spans from fish (infected by *A. invidans*), to crayfish (infected by *A. astaci*), to plants such as beet, legumes, and radish (infected by *A. cochlioides*, *A. euteiches*, and *A. raphani*, respectively). In the crayfish pathogen *A. astaci*, secreted protease activity has been implicated in the penetration of the host cuticle during pathogen invasion (Dieguez-Uribeondo and Cerenius, 1997). In the present report, a preliminary examination was undertaken to determine the presence and forms of protease secreted by the phytopathogens *A. cochlioides* and *A. euteiches*. Presence of abundant protease activity of apparent pathogen origin in infected plant tissues in both pathosystems implicates protease activity in Aphanomyces virulence.

MATERIALS AND METHODS

Isolates 19-1z of *A. cochlioides* and MM 174 of *A. euteiches* (generously provided by C. Windels, University of Minnesota at Crookston) used in the study were maintained on corn meal agar and stored in oatmeal broth (Parke and Grau, 1992). Substrate for the production of protease activity consisted of 30 ml sterile deionized, distilled water containing autoclaved pieces of either sugarbeet root slices (for *A. cochlioides*) or pea epicotyls (for *A. euteiches*) in glass Petri dishes. After inoculation of media with hyphal plugs of the respective pathogens, the plates were sealed with Parafilm and incubated in the dark at 22ºC. Cuture supernatants were harvest at 14 days post-inoculation, filtered through Miracloth to remove mycelial debris, and stored at -20ºC. Analysis of protease activity in infected pea (*Pisum sativum* cv. 'Wando') and sugarbeet (*Beta vulgaris* cv 'Ultramono') seedlings was done after inoculation of emerged seedlings with zoospore suspensions. Extracts were made from seedlings just as water-soaking symptoms were appearing in the tissue. Infected tissue was expressed in a microfuge tube containing distilled water by grinding with a microfuge pestle at a rate of ~10 ml water per gram fresh weight tissue. Extracts were clarified by centrifugation at 14,000 x g for 5 min. in a microfuge and the supernatant was stored at -20ºC until further use.

Bulk protease activity was assayed as the digestion of azocasein thereby liberating a non-precipitable dye that absorbs at 440 nm (Sarath et al., 2001). Reactions consisted of a 10X concentrate of the bulk supernatants (30 ul) combined with 60 ul of 2% azocasein dissolved in 0.1M TRIS-HCl pH 8.0. Reactions were incubated at 37ºC overnight with or without the inclusion of protease inhibitor followed by the addition of 3 vol. of 10% trichloroacetic acid. Precipitated, undigested azocasein was removed by centrifugation, the supernatant was adjusted to 0.5M NaOH, and the absorbance of each sample determined at 440 nm. Values presented for each sample represent the average of reactions performed in triplicate.

Separation of protease isozymes on polyacrylamide gels was done essentially as described by Sarath et al. (2001). Culture supernatants were mixed with standard SDS-PAGE loading buffer (lacking β-mercaptoethanol) and loaded without heating directly onto gels impregnated with gelatin. After electrophoresis, gels were incubated for 1 hr. in a 1% TRITON-X100 solution to remove the SDS and then incubated at 37ºC overnight in a solution containing 1% TRITON X-100, 0.1M TRIS pH 8.0 and 1 mM $CaCl_2$. Protease inhibitors were incorporated either directly into the gel (for proteinacious inhibitors) or into the incubation buffer for inhibition experiments. Gels subsequently were stained with Coomassie brilliant blue, destained in 10% each of MeOH and acetic acid, and dried onto Whatmann 3MM paper. Gels were scanned with a UMAX Vista S12 flatbed scanner for documentation.

RESULTS AND DISCUSSION

Bulk assays of protease activity in culture supernatants of *A. cochlioides* indicated that much of the activity was of a trypsin class (Table 1). Thus antipain, leupeptin, chymostatin, and aprotinin (as well as the broad-spectrum Pefabloc) reduced the digestion of azocasein by more than 2.5X that of inhibitors that had no effect (i.e. pepstatin, bestatin, and EDTA). This correlates with the expression by *A. astaci* of serine proteases of the subtilisin and trypsin class, proteins whose genes were recently cloned and characterized (Bangyeekhun et al., 2001).

Although bulk assays yield quantitative estimates for protease activity, the forms of protease are best determined by isozyme analysis. Gel electrophoresis of culture supernatants and infected plants clearly demonstrated that proteases secreted by *A. cochlioides* and *A. euteiches* are comprised of multiple isozymes (Figs. 1 and 3). Eight isozyme bands were resolved by SDS-PAGE for supernatants from *A. cochlioides* whereas six isozymes from *A. euteiches* supernatants were observed. The differences in the migration of the isozymes suggest that protease zymography may be useful in distinguishing Aphanomyces species from each other. This could be tested within *A. euteiches*, where different DNA polymorphic types have been characterized, and between *A. euteiches* and other phytopathogenic Aphanomyces.

Zymograms also provide an elegant means to identify protease class amonst the isozymes displayed. By incorporating trypsin inhibitors directly into the activity assay, the fastest migrating proteases of *A. cochlioides* were shown to be of a trypsin class (Figure 2). Partial inhibition of the medium migrating protease activity was observed in the presence of serine protease inhibitors, suggesting that it may be this class as well. Interestingly, whereas the lima bean trypsin inhibitor (LBTI) provides potent inhibition of the fast migrating proteases from *A. cochlioides*, this protein fails to appreciabley inhibit any of the protease isozymes produced by *A. euteiches* (Figure 3A). Nonetheless, the fast migrating isozyme(s) of *A. euteiches* is indeed a serine protease as demonstrated by its inhibition by Pefabloc (Figure 3B). It is tempting to speculate that a homolog of LBTI exists within legumes that are a host for *A. euteiches* and that serine proteases in this

organism have evolved to evade inhibition. This could be tested by eventually cloning the genes for the proteases produced by *A. euteiches* and transferring them to the genome of *A. cochlioides*, which induces minimal disease on inoculated legumes.

New technologies exist in laboratories today for dissecting host-pathogen interactions in plant pathology. The analysis of the involvement of protease and protease inhibitors as host range determinants in the phytopathogenic Aphanomyces will benefit from technologies applied from both the host and pathogen side. Cloning of the genes encoding proteases in Aphanomyces may lead to the disruption of these genes by RNA interference or, exploiting the homothallic nature of this oomycete, by insertional mutagenesis. This will reveal more precisely their role in pathogenesis or virulence. Transfer of protease genes between species will elucidate their potential role as host range determinants. Should protease inhibition constitute a basis for plant resistance to Aphanomyces, genes encoding protease inhibitors may co-segregate with QTL for disease resistance. The cloning of candidate inhibitor genes, assay of their encoded proteins against proteases produced by Aphanomyces, and their knock-out using RNA silencing eventually would permit determination of the role of protease in the induction of Aphanomyces root rot disease and of protease inhibitors in plant defense.

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Table 1. Inhibition of *A. cochlioides* protease activity by serine protease inhibitors.

Source[a]	Inhibitor or Commercial Enzyme[b]	O.D.$_{440}$[c]
Extract +	Antipain	0.047
	Bestatin	0.122
	Chymostatin	0.073
	E-64	0.097
	Leupeptin	0.057
	Pepstatin	0.13
	Phosphoramidon	0.13
	Pefabloc	0.038
	EDTA-Na_2	0.131
	Aprotinin	0.059
Commercial	Trypsin 25 ug/ml	0.583
	Trypsin 25 ug/ml	0.625
	Trypsin 25 ug/ml + LB inhibitor 75 ug/m	0.209
	Trypsin 25 ug/ml + LB inhibitor 75 ug/m	0.207

[a]Either extracts prepared from cultured *A. cochlioides* or a commercial source of trypsin were used (Sigma Chemical, St. Louis, MO).
[b]Inhibitors were tested at concentrations recommended by the supplier (Boehringer-Mannheim Biochemical, Mannheim, Germany). Underlined inhibitors act against serine proteases.
[c]Optical density recorded at $\lambda = 440$ nm is an average of triplicate reactions.

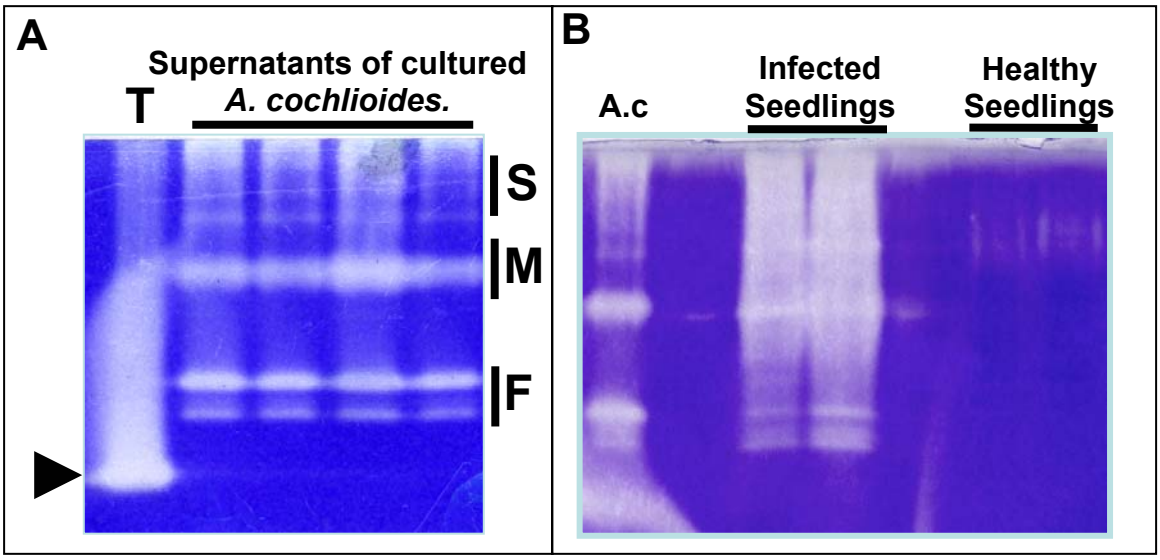

Figure 1. Secretion of protease activity by *A. cochlioides* (A) and accumulation of protease activity in infected sugarbeet seedlings (B). Culture supernatants were separated by SDS-PAGE under non-denaturing conditions in the presence of co-polymerized gelatin. After proteolysis, gels were stained with Coomassie brilliant blue and digitally recorded on a flat-bed color scanner. Commerical trypsin (T) is included on the gel as a control. The *A. cochlioides* proteases are broadly divided into those possessing a fast (F), medium (M), and slow (S) migration in the gel. In B, culture supernatant of A. cochlioides (A.c.) is run for comparison.

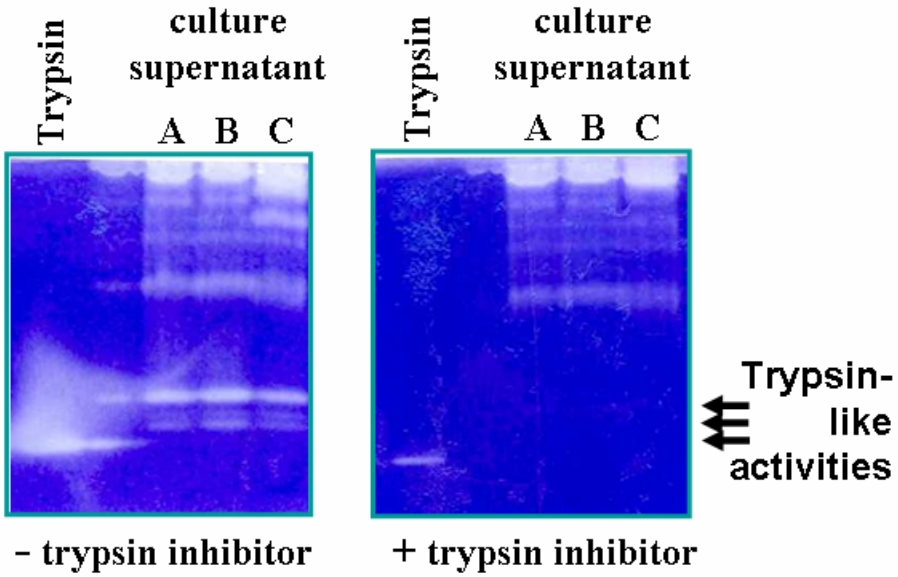

Figure 2. Inhibition of *A. cochlioides* protease activity by lima bean trypsin inhibitor (LBTI). Gels co-polymerized with gelatin (both panels) and with gelatin + LBTI were allowed to digest followed by staining with Coomassie brilliant blue and recording with a color scanner. Note the decreased intesity of digestion for both the commerical trypsin and the fast migrating *A. cochlioides* isozymes.

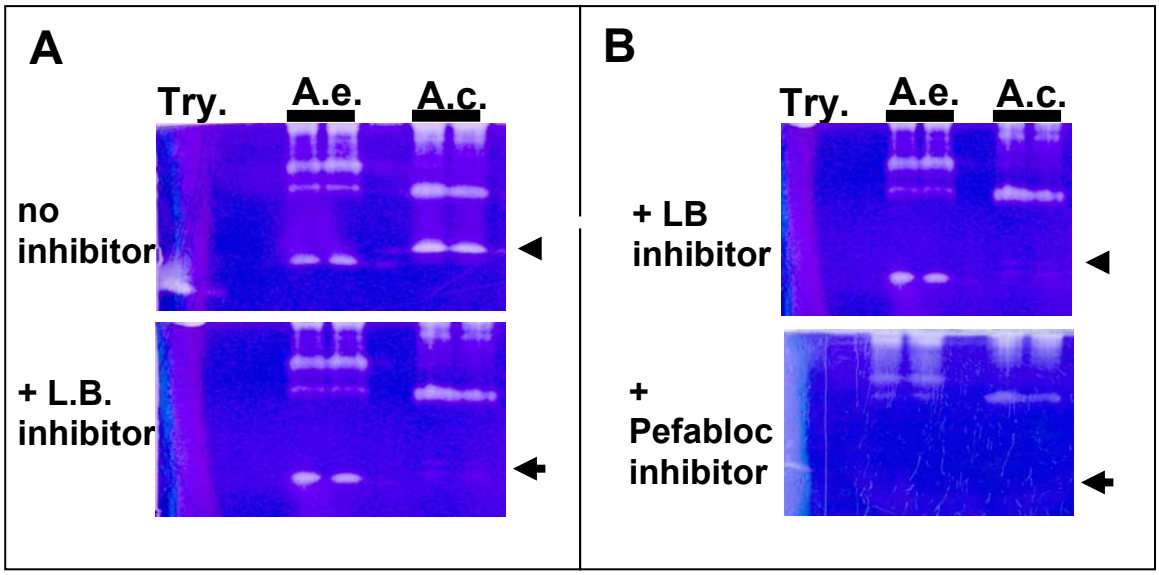

Figure 3 Differential inhibition of *A. cochlioides* and *A. euteiches* proteases by lima bean trypsin inhibitor (LBTI). In A, the gelatin digestion in the PAG is tested without inhibitor or with LBTI present. In B, the difference in inhibition between gels containing LBTI and Pefabloc is shown. Note the insensitivity of the *A. euteiches* fast migration isozyme to LBTI.

Oomycete genomics: Status and prospects for *Aphanomyces* research

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SUMMARY

The oomycetes form a unique lineage of stramenopile eukaryotes, unrelated to true fungi, but closely related to heterokont (brown) algae. It is clear that oomycetes evolved the ability to infect plants independently from other eukaryotic plant pathogens, mainly the "true" fungi, and are likely to have unique mechanisms to do so. In this talk, I will provide an update on genomic resources for *Phytophthora* and other oomycete species, summarize the applications of genomics to oomycetes with particular reference to functional genomics of *Phytophthora infestans*, and, finally, discuss how a genome project would impact research on *Aphanomyces* and how such a project could be undertaken.

INTRODUCTION

The oomycetes represent a diverse group of organisms that includes pathogens of plants and animals, as well as saprophytic species (water molds). The oomycetes form a unique lineage of stramenopile eukaryotes, unrelated to true fungi, but closely related to heterokont (brown) algae. This is has been well established using molecular phylogenies based on ribosomal RNA sequences, compiled amino acid data for mitochondrial proteins, and several protein encoding chromosomal genes. It is evident from these analyses that oomycetes evolved the ability to infect plants independently from other eukaryotic plant pathogens, mainly the "true" fungi, and are likely to have unique mechanisms to do so.

Phylogenetic analyses based on sequence data also contributed to our understanding of the evolution of plant pathogenesis within the oomycetes. The ability to infect plants has evolved at least twice in the oomycete lineage. First, in the ancient monophyletic group that is comprised of the majority of plant pathogenic genera, such as *Phytophthora, Pythium, Perosnospora*, *etc...* Second, in the genus *Aphanomyces*, which is more closely related to the Saprolegniales and includes both animal and plant pathogenic species. In the future, comparative genomic analyses between *Apahanomyces* and other plant pathogenic oomycetes, as well as between plant and animal pathogenic oomycetes should help define the basic set of pathogenicity genes that allow oomycetes to infect dicot plants.

In this talk, I will provide an update on genomic resources for *Phytophthora* and other oomycete species, summarize the applications of genomics to oomycetes with particular reference to functional genomics of *Phytophthora infestans*, and, finally,

discuss how a genome project would impact research on *Aphanomyces* and how such a project could be undertaken.

RESULTS & DISCUSSION

Structural genomic studies of oomycetes are well under way within the framework of various research consortia, as well as efforts in individual laboratories. A number of ongoing and pending projects focus on *P. infestans*, *Phytophthora sojae*, and several other *Phytophthora* species. Projects on other oomycete species, include sequencing of cDNAs from the biotrophic downy mildews *Peronospora parasitica*, *Bremia lactucae*, and *Plasmopara halstedii*, as well as from the fish pathogen *Saprolegnia parasitica*.

Genome science promises to significantly impact our understanding of oomycete biology and pathology. The main applications of oomycete genomics are:

- Understanding the molecular basis of pathogenicity through the identification of genes that contribute to the infection process.
- Identifying targets for genetic control, specifically avirulence genes that function at host and nonhost level.
- Identifying targets for chemical control, such as essential genes.
- Understanding population structure and evolution, through improved molecular markers and markers that assay for phenotypes.

A genome project would greatly impact research on *Aphanomyces.* A first stage in such a project would be to generate random cDNA sequences or expressed sequence tags (ESTs). An EST data set would impact research beyond basic studies. ESTs will facilitate the identification and development of genetic markers, such as single nucleotide polymorphisnms (SNPs), and will also allow novel diagnostic tools. In addition, the availability of *Aphanomyces* sequence data means that this organism will be incorporated in comparative genomic analyses within the oomycetes and between oomycetes and other microbial pathogens. This will result in significant insight into the biology and pathology of *Aphanomyces* and will unravel totally unsuspected mechanisms

An EST project for Aphanomyces would consist of three steps. (1) Construction of cDNA libraries from various developmental and infection stages; (2) sequencing of random cDNAs; and (3) bioinformatic processing of sequence data and annotation of sequences. All of these steps can be outsourced to companies or specialized institutions. For example, the EST processing platform, known as XGI, developed by the National Center for Genome Resources (www.ncgr.org) can be used to process ESTs and allow the dissemination of the sequence data to the research community through the internet. NCGR currently hosts the Phytophthora Functional Genomics Database (PFGD, www.pfgd.org).

The cost of an Aphanomyces EST project will obviously depend on the scale and sequencing depth. Nevertheless, based on analysis of the Phytophthora EST data, it is

clear that even a few thousand ESTs can provide enormously useful information and insight into the biology of the organism. Given the current cost of cDNA sequencing (sequencing 1,000 ESTs costs approximately $6,000), an EST project for Aphanomyces could be initiated with minimal investment. Indeed, my laboratory is collaborating with the CNRS and University Paul Sabatier, Toulouse, France, unit "Cell surface in phytopathogenic fungi/oomycetes, B. Dumas and collaborators" to initiate an EST project for Aphanomyces euteiches.

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Real time PCR assays for examining resistance to *Aphanomyces euteiches* and *Phytophthora medicaginis* in legumes and for studying microbial population dynamics in mixed plant infections

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SUMMARY

A real-time PCR assay was used to quantify the relationship in alfalfa and pea between disease severity and amount of *Aphanomyces euteiches* detected in roots. Spearman rank correlations between pathogen DNA content and disease severity index (DSI) ratings were positive and significant for both alfalfa and pea. In all experiments, significantly more pathogen was detected in susceptible populations than in resistant populations.

Another real time PCR assay was developed to quantify the amount of *Phytophthora medicaginis* DNA in alfalfa plants. Significantly less pathogen DNA was detected in bulked samples of a highly resistant check population than in bulked samples from moderately resistant and susceptible check populations. The Spearman rank correlation between pathogen DNA content and the number of resistant plants in a bulked sample was negative and significant. Analysis of individual plants indicated that significantly less pathogen DNA was detected in resistant plants than in susceptible plants.

These results clearly demonstrate that reduced pathogen colonization is characteristic of the resistance response in alfalfa and peas to two different Oomycete plant pathogens. The two real-time assays may be useful for simultaneously selecting for resistance to both pathogens and for studying microbial population dynamics in mixed plant infections.

INTRODUCTION

Aphanomyces euteiches Dreschs. causes root rot of several globally important legume crops, including alfalfa (*Medicago sativa* L.) (Grau, 1990) and pea (*Pisum sativum* L.) (Pfender, 2001). No effective chemical control for this disease of alfalfa is available. To minimize losses, the cultivation of resistant alfalfa cultivars is recommended, along with the avoidance of poorly drained and heavily infested fields (Grau, 1990). Root rot of pea caused by *Aphanomyces euteiches* Dreschs. is one of the most yield limiting diseases associated with pea production. Losses due to Aphanomyces root rot can be managed by avoidance of heavily infested fields and the cultivation of cultivars that have at least tolerance to *A. euetiches* (Pfender, 2001).

The majority of alfalfa and pea breeding programs have used an integer scale to score severity of disease caused by *A. euteiches*. The most widely accepted method for evaluating resistance in alfalfa uses a scale of 1 to 5 for evaluating disease severity, where 1 = healthy plant and 5 = dead plant (Fitzpatrick *et al.*, 1998). Similarly, many pea

breeding programs use integer scales to rate disease severity. The primary constraint in the use of an integer rating scale is the unavoidable loss in accuracy that accompanies a system where a continuous range of disease resistance is scored in discrete classes. An alternative method for evaluating disease resistance that more precisely quantifies disease would afford breeders greater ability to discriminate between plants that appear to be phenotypically similar based on the visual assessment of disease severity.

Recently, Vandemark *et al*. (2002) developed a real-time quantitative PCR assay for quantifying the amount of *A. euteiches* DNA in infected alfalfa. Analysis of individual plants and bulked plant samples of several alfalfa populations resulted in Spearman rank correlations between pathogen DNA content and disease severity index (DSI) ratings that were positive and significant. These results suggested that it may be possible to use this real time quantitative PCR assay for selecting the most resistant plants among a sample of plants that are indistinguishable based on a visual assessment of disease severity.

We are interested in developing real time fluorescent PCR assays for other alfalfa pathogens, including *Phytophthora medicaginis* Hansen et Maxwell. *P. medicaginis* has been implicated in damping-off disease complexes of alfalfa with *A. euteiches* (Havey and Grau, 1985; Munkvold and Carlton, 1995). Field studies have indicated that alfalfa cultivars with resistance to both *A. euteiches* and *P. medicaginis* have significantly higher yields and greater plant vigor than cultivars that lack dual resistance (Holub and Grau, 1990; Wiersma *et al*., 1995). Quantitative PCR assays specific for both *A. euteiches* and *P. medicaginis* could be used in multiplex assays for simultaneously quantifying the amount of both pathogens in infected alfalfa. This would provide a means for simultaneously selecting plants for resistance to both *A. euteiches* and *P. medicaginis*, and also provide tools for examining microbial population dynamics in plants infected with both pathogens.

The objectives of this study were: 1) apply the real-time quantitative PCR assay to examine the relationship between disease severity and the amount of *A. euteiches* DNA detected in roots of infected alfalfa plants using additional, previously unexamined, pathogen isolates, 2) employ the assay to examine the relationship between disease severity and the amount of *A. euteiches* DNA detected in roots of infected pea plants, and 3) develop a real-time quantitative PCR assay that was specific for *P. medicaginis*.

MATERIALS AND METHODS

Inoculations and evaluation of disease severity in alfalfa. The standard test protocol for evaluating resistance in alfalfa to *A. euteiches* (Fitzpatrick *et al*., 1998) was followed in this study. Alfalfa seedlings were inoculated with zoospores of *A. euteiches* MW5-43 (race 1) or *A. euteiches* WI98 (race 2). Disease severity index (DSI) ratings were done on the surviving seedlings 14 days after inoculations using an integer scale from 1-4 as follows: 1 = no necrosis of roots and hypocotyls; 2 = slight necrosis of roots and hypocotyls; 3 = necrosis of roots and lower hypocotyl, slight chlorosis of cotyledons, and

moderate stunting of stem, and 4 = extensive necrosis of roots, hypocotyls and cotyledons, and severe stunting of stem (Fitzpatrick *et al*., 1998).

Inoculations and evaluation of disease severity in pea. Pea seedlings of the cultivar Bolero (susceptible) and the germplasm 90-2079 (tolerant) were inoculated with 25000 zoospores each of *A. euteiches* isolate SP7. Fourteen days after inoculations, plants were visually scored for disease severity using a scale of 0-5 as follows: 0 = no visible symptoms; 1 = a few small discolored lesions on the entire root system; 2 = minor discoloration covering of root system; 3 = brown discoloration on entire root system, no symptoms on epicotyl or hypocotyl; 4 = brown discoloration on entire root system, shriveled and brown epicotyl or hypocotyls, and 5 = dead plant (Davis *et al*., 1995).

Quantitation of *A. euteiches* DNA in infected plants. DNA isolated from infected plants was analyzed using the primer/probe set 136F-161T-211R (Vandemark *et al.,* 2002). PCR for each plant sample was performed in 50 µl reactions containing 100 ng DNA. For the quantification of *A. euteiches* isolate MW5-43 (race 1), *A. euteiches* isolate WI-98 (race 2), and *A. euteiches* isolate SP7, standard curves were constructed using pure DNA of the respective isolates.

Design of a real-time PCR assay specific for *P. medicaginis*. A sequence characterized DNA marker (SCAR) that was only amplified in isolates of *P. medicaginis* was analyzed using Primer Express software (Applied Biosystems, Foster City, CA) to identify candidate sequences for PCR primers and probes. The 5′ terminus of the probe (TaqMan; Applied Biosystems) was labeled with the fluorochrome 6-carboxyfluorescein (6FAM) and the 3' terminus labeled with the quencher dye tetra-methylcarboxyrhodamine (TAMRA). The primer/probe set amplified a 61 bp fragment.

Inoculations of alfalfa with *P. medicaginis* and evaluation of disease severity. The standard test protocol for evaluating seedling resistance in alfalfa to *P. medicaginis* was closely followed in this study (Nygaard et al., 1995). Three different standard check alfalfa populations were evaluated: the varieties Saranac (susceptible) and Agate (resistant), and the germplasm WAPH-1 (high resistance).

Ten days after inoculation, plants were rated for resistance as follows: resistant = vigorously growing plants with slight to no necrosis of tap and secondary roots; hypocotyls area sound with slight to no chlorosis of cotyledons, or susceptible = stunted or dead plant, severe necrosis of roots, hypocotyl and cotyledons (Nygaard *et al.,* 1995).

Quantitation of *P. medicaginis* DNA in infected plants. DNA isolated from infected plants was analyzed using the primer/probe set. For each DNA sample, four replicate reactions were run in 50 µl reactions containing 100 ng of DNA, 900 nM forward primer, 900 nM reverse primer, 150 nM probe, 5 µl ddH_2O, and 25 µl of 2X TaqMan Universal

PCR Master Mix (Applied Biosystems). Amplifications and detection of fluorescence were done using a GeneAmp 7000 Sequence Detection System (Applied Biosystems).

RESULTS

PCR analysis of alfalfa plants infected with *A. euteiches.* A comparison of means for the amount of MW5-43 (race 1) and WI-98 (race 2) detected in plants of each DSI class is presented (Table 1). For both isolates, significant differences existed between DSI classes (1-4) for the amount of pathogen DNA detected ($P < 0.05$). For both isolates, the correlation between DSI and the amount of pathogen DNA detected in plants was positive and significant (Table 1).

TABLE 1. Comparison of means for quantity (ng) of *Aphanomyces euteiches* DNA in plants with different disease severity index (DSI) ratings of resistant standard check alfalfa populations WAPH-1 and WAPH-5[a].

DSI	*A. euteiches* MW5-43 (WAPH-1)	*A. euteiches* WI-98 (WAPH-5)
1	1.36 a	1.97 a
2	3.04 b	2.00 a
3	8.22 c	2.51 b
4	8.34 c	3.79 c
LSD[b]	0.93	0.44
P (P > |ρ|)	0.78 (<0.0001)	0.60 (<0.0001)

[a]N = 12 plants for each DSI rating. DNA was extracted from roots of single plants and tested using primer/probe set 136F-161T-211R. Each plant was tested with three replicate PCR reactions. Ratings: 1-4 as described in Materials and Methods.The Spearman rank correlation (ρ) between DSI and pathogen DNA quantity is presented.

[b] LSD = Least significant difference ($\alpha = 0.05$). Means within a column followed by the same letter are not significantly different.

PCR analysis of pea plants infected with *A. euteiches.* Results of comparisons of means between 90-2079 and Bolero for the amount of pathogen DNA detected in roots of infected plants are presented (Table 2). Significantly less SP7 DNA was detected in 90-2079 than in the susceptible cultivar Bolero. The correlation between DSI rating of a plant and the amount of *A. euteiches* SP7 DNA detected in the plant was positive and significant (Table 2).

TABLE 2. Comparison of means[a] between pea plants infected with *A. euteiches* SP7 for disease severity index (DSI) ratings[c] and quantity (ng) of *Aphanomyces euteiches* DNA[a].

Population	ng DNA	DSI
90-2079 (tolerant)	4.86 a	3.54 a

Bolero (susceptible)	11.24 b	4.79 b
LSD ($\alpha = 0.05$)[b]	1.81	0.32
ρ (Prob > $\lvert\rho\rvert$)	0.57 (< 0.0001)	

[a]The Spearman rank correlation (ρ) between DSI and pathogen DNA quantity is presented. N = 24 plants of each population. DSI ratings: 1-5 as described in Materials and Methods. DNA extracted from the entire root system of each plant was tested using the primer/probe set 136F-161T-211R in three replicate PCR reactions.

[b]LSD = least significant difference ($\alpha = 0.05$). Means within a column followed by the same letter are not significantly different.

Discriminating between alfalfa standard check populations for resistance to *P. medicaginis*. Standard curves were generated for the primer/probe set using DNA isolated from several different isolates of *P. medicaginis*. Correlations between initial DNA quantity and C_T value exceeded – 0.99 for all isolates and the primer/probe set did not amplify DNA from any other tested organisms, including alfalfa populations and several other Oomycete plant pathogens. These results indicate that the primer/probe set precisely and specifically amplified *P. medicaginis* DNA.

Significant differences were observed for the amount of DNA of *P. medicaginis* WI301 detected in bulked plant samples of the susceptible check Saranac, the resistant check Agate, and the highly resistant check WAPH-1. Significantly less pathogen DNA was detected in the highly resistant check WAPH-1 than was detected in the two other check populations. Significantly less DNA was detected in the resistant check Agate than was detected in the susceptible check Saranac. The Spearman rank correlation between the number of resistant plants in a bulk sample and the amount of *P. medicaginis* WI301 DNA detected in the bulk sample was negative and significant for both experiments.

DISCUSSION

The results presented in this report demonstrate the specificity and accuracy of a method for quantifying the amount of *A. euteiches* in infected alfalfa and pea plants based on the detection of a fluorescent-labeled amplicon produced by a PCR reaction. Similarly, results also demonstrate the specificity and accuracy of a method for quantifying the amount of *P. medicaginis* in infected alfalfa. Correlations between disease severity and the amount of pathogen DNA were positive and significant for both *A. euteiches* and *P. medicaginis*. This suggests that resistance to these pathogens is characterized by an inhibition of pathogen multiplication in infected tissues.

It should be possible to use the real time PCR assay for *P. medicaginis* in combination with the real time PCR assay for *A. euteiches* to accurately identify plants with high levels of resistance to both pathogens. The real time PCR assays that are specific for *P. medicaginis* and *A. euteiches* may also facilitate the study of factors, both abiotic and biotic, which influence microbial population dynamics in roots that are

simultaneously or serially infected with both pathogens. The alfalfa populations WAPH-1 and Saranac are the highly resistant and susceptible standard alfalfa checks respectively for both *P. medicaginis* (Nygaard *et al*., 1995) and *A. euteiches* race 1 (Fitzpatrick *et al*., 1998). These two populations would be appropriate plant materials for studying interactions between *P. medicaginis* and *A. euteiches* in resistant and susceptible plants.

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Within field phenotypic and genotypic diversity in *Aphanomyces euteiches*

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SUMMARY

Our objectives were to assess the degree of variation for genotype and pathogenicity in a population of *Aphanomyces euteiches* within two grower's fields with a history of Aphanomyces root rot. We evaluated two hypotheses: (1) populations of *A. euteiches* are diverse genotypically and phenotypically within single fields, and (2) that populations of *A. euteiches* from different fields are well differentiated. Genotypic diversity was determined using amplified fragment length polymorphism fingerprinting while phenotypic diversity was based on greenhouse studies to determine disease severity readings on 5 pea clones differing in resistance to *A. euteiches*. Populations of *A. euteiches* recovered from two fields in northeast OR and western WA are genetically diverse within a field, yet well differentiated when comparing both fields. At the same time, both populations are well adapted to their pea hosts and are very aggressive to the five pea lines included in this study.

INTRODUCTION

Our objectives were to assess the degree of variation for genotype and pathogenicity in a population of *Aphanomyces euteiches* within two grower's fields with a history of Aphanomyces root rot. We evaluated two hypotheses: (1) populations of *A. euteiches* are diverse genotypically and phenotypically within single fields, and (2) that populations of *A. euteiches* from different fields are well differentiated. Genotypic diversity was determined using amplified fragment length polymorphism fingerprinting while phenotypic diversity was based on greenhouse studies to determine disease severity readings on 5 pea clones differing in resistance to *A. euteiches*.

MATERIALS & METHODS

Soil sampling. A total of 8-10 soil samples of approximately a gallon each were collected from two agricultural fields that had a history of Aphanomyces root rot. The first field was located near Athena, OR (NE-OR). The second field was located in Eastern Washington near Mt. Vernon (W-WA). These two locations are physically separated by the Cascade Mountains. Both fields had a history of Aphanomyces root rot. A total of 10-15 isolates were recovered from each individual soil sample, and 8-10 soil samples were collected per location.

***Aphanomyces* isolations.** Isolates were obtained using the wet-sieving/baiting technique described previously (Kraft, Marcinkowska, and Muehlbauer 1990). Briefly, a 100-g subsample of each soil sample was screened through a 10-mesh screen (2-mm opening) and homogenized for 3 min. in a blender with 500 ml sterile distilled water. The organic debris was recovered by sieving with a 200-mesh sieve (75 μm opening), flotation of the debris to separate it from mineral particles followed by a final sieving step (200-mesh) to recover the organic debris. The moist organic fraction was stored at 4° C until baited.

Five-day old pea-seedlings (cv. Bolero) were used to bait *A. euteiches* out of the organic debris. Surface-disinfested seed were germinated on sterilized germination paper at room temperature under 16-hr day fluorescent lighting. Approximately 10 mm^3 of debris were placed on the taproot of each seedling. *A. euteiches* was recovered by plating a surface sterilized root section on corn meal agar. Isolates were maintained on cornmeal agar or as agar plugs in sterile water.

Pathogenic phenotype determination. A selection of five breeding lines/cultivars consisting of PI166159, PI180693, 79-2022, Bolero and DSP were used to describe variation in phenotype for host aggressiveness. Twelve seed per line were planted in a row in perlite trays. Seed was surface sterilized with 10% bleach. All five lines were planted in a tray and each tray was inoculated with one isolate. Two replications were used per experiment and the experiment was repeated twice. Plants were scored 4 weeks after planting on a 0 (= no symptoms) to 5 (= plants dead) scale.

Inoculum of *Aphanomyces* was prepared in pea broth (10g DSP seed in 200ml distilled water in 500ml Erlenmeyer flask) 5-7d after emergence of pea plants. Pea broth was inoculated with an agar-plug of an actively growing CMA colony and incubated at room temperature for 6 days. Mycelial mats were removed, rinsed 3 times in distilled water before incubation in 250 ml salt solution (Mitchell and Yang 1966) overnight with forced aeration. Zoospore concentration was adjusted to 10^5 spores/ml. Plants were dip-inoculated in 5 ml of zoospore solution. Uninoculated control plants were always included as a control treatment.

Isolation of genomic DNA. Strains were grown in potato dextrose broth (Difco Laboratories, Detroit, MI) at room temperature for 7-10 days. DNA was isolated using the FastDNA kit (QBiogene, Carlsbad, CA).

Fluorescent amplified fragment length polymorphisms (FAFLP). FAFLP (Vos et al. 1995) was performed on genomic DNA of *A. euteiches* using the AFLP Microbial Fingerprinting protocol (PE Applied Biosystems, Foster City, CA) with slight modifications. DNA (20 ng) was digested and adaptors ligated in a 11 μl reaction volume with *Eco*RI (10 U), *Mse*I (50 U), T4 DNA ligase (10 Weiss units), 1.0 μl *Mse*I/*Eco*RI adaptor, 1.1 μl 0.5 M NaCl, 0.5 μl BSA (1.0 mg/ml), and 1.1 μl 10X T4 DNA ligase buffer (New England Biolabs, Beverly, MA; 50mM Tris-HCl (ph 7.8), 10mM $MgCl_2$, 10 mM dithiothreitol, 1mM ATP, 25 μg/ml BSA) for 2 hours at 37º C. Restriction-ligation samples were diluted with 189 μl TE buffer (20mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Preselective amplifications of 4.0 μl restriction-ligation sample with 0.5 μl *Eco*RI core primer, 0.5 μl *Mse*I core primer and 15 μl AFLP Amplification

Core Mix (PE Applied Biosystems) were performed on a GeneAmp 9700 thermal cycler (PE Applied Biosystems) programmed as follows: 2 min at 72º C; 20 cycles of 20 sec at 94º C, 30 sec at 56º C, and 2 min. at 72º C; and cooling to 4º C. Amplicons (10 µl) were checked on 1.2% agarose gels (4V/cm for 3-4 hrs.) in 1X TBE buffer and visualized with ethidium bromide and UV illumination. Ten µl pre-amplified samples weree diluted with 190 µl TE buffer. Selective PCR was performed on 1.5 µl diluted, pre-amplified product in a 10µl reaction volume with 0.5 µl *Mse*I-CT primer at 5µM, dye-labeled 0.5 µl *Eco*RI-AT primer at 1µM and 7.5 µl AFLP Core Amplification Mix (Applied Biosystems). Reactions were performed under the following conditions: 2 min at 94º C; 10 cycles of 20 sec at 94º C, 30 sec at 66º C and 2 min at 72º C (annealing temperature are lowered by 1º C during each cycle); followed by 20 cycles of 20 sec at 94º C, 30 sec at 56º C, and 2 min. at 72º C; and a final extension for 30 min at 60º C and cooling to 4º C. Products (0.5 µl) were run with 25 µl loading buffer (24 µl deionized formamide and 0.05 µl GeneScan-500 size standard; PE Applied Biosystems) on a capillary sequencer (ABI Prism 310). Samples were run in a 47cm/50 µm capillary with POP-4 polymer. Samples were injected for 12 sec at 15 kV and run at 13 kV for 34 min at 60ºC.

Statistical analysis. AFLP patterns were analyzed using GeneScan and Genotyper software (Applied Biosystems), manually correcting for controversial bands. Bands were treated as putative single AFLP loci and a binary matrix containing the presence or absence of these reproducible bands was constructed and used for further analysis. Genotypes are defined as multilocus genotypes using all AFLP loci scored (Grünwald et al. 2003; Grünwald et al. 2001). Statistical analyses were conducted with POPGENE 1.32, available at no cost from the University of Alberta, Canada and TFPGA (Tools for Population Genetic Analyses, version 1.3, available at no cost from Mark P. Miller at Northern Arizona University, Flagstaff, AZ). Each AFLP band was assumed to represent the dominant genotype at a single locus while the absence of that same band represents the alternate homozygous recessive genotype. We assumed a diploid model with 2 alleles per locus and estimated the frequency of the recessive allele by a Taylor expansion estimator (Lynch and Milligan 1994). Genotypic diversity analysis was used to determine the distribution of genetic diversity among populations (Grünwald et al. 2003; Grünwald et al. 2001). Heterozygosity was estimated for populations and subpopulations. Genotypic diversity was calculated using Shannon-Wiener's index *H'* (Grünwald et al. 2003; Shannon and Weaver 1949). Pair-wise measures of Rogers' modified genetic distance and population differentiation using Nei's coefficient of differentiation (G_{ST}) (Nei 1978; Slatkin and Barton 1989) were calculated using POPGENE. Cluster analysis of multilocus AFLP genotypes was based on allele frequencies observed for each population. A phenogram was constructed using the unweighted pair-group method of averages (UPGMA) algorithm from a Rogers' modified genetic distance matrix. Bootstrap sampling (1000 replicates) was performed for parsimony analysis of the constructed phenogram (Felsenstein 1985).

RESULTS & DISCUSSION

Isolates retrieved from individual soil samples from the same field in NE Oregon

differed little in pathogenicity (Figure 1). The population of *A. euteiches* present in this field is well adapted to pea as a host. Clone 180693 was the most resistant clone evaluated.

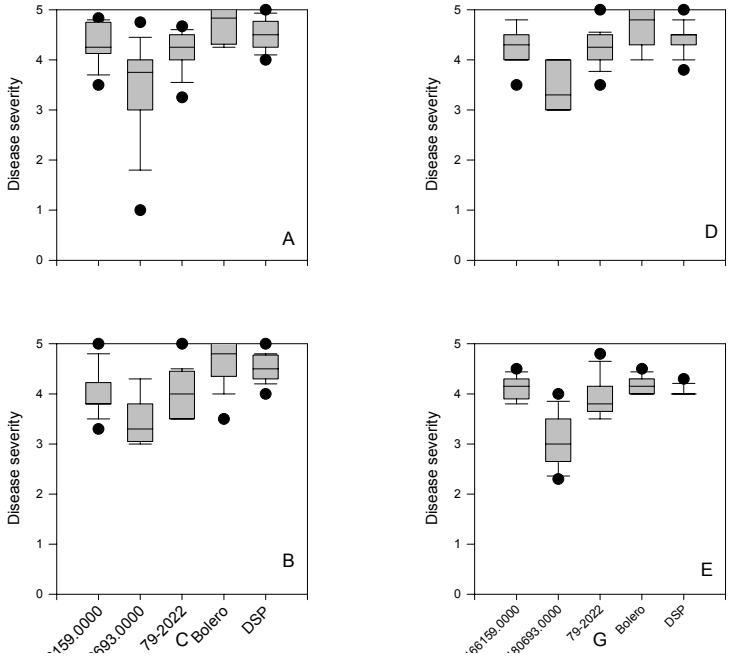

Figure 1. Phenotypic diversity for subpopulations (10-15 isolates per soil sample) *Aphanomyces euteiches* retrieved from 4 individual soil samples from NE-OR: A, B, D, and E.

Populations of *A. euteiches* within a soil smple were genotypically diverse, although clones of the same genotype could be recovered (Table 1). This observation was similar in nature for samples obtained in W-WA and NE-OR. Gene diversity ranged between 0.08-0.22 for individual soil samples while genotypic diversity ranged between 1.7 and 2.5 (Table 1).

Table 1. Sample size (*n*), number of observed multilocus genotypes (*g*), Nei's gene diversity (*h*) (Nei 1973) and multilocus genotypic diversity (H') (Grünwald et al. 2003; Shannon and Weaver 1949) for populations of *Aphanomyces euteiches* within two soil samples taken each East and West of the Cascade Mountains.

Location	Soil sample #	*n*	*g*	*h*	*H'*
East	2	16	13	0.201	2.48
East	4	14	7	0.121	1.67
West	14	11	11	0.216	2.40
West	15	14	8	0.081	1.91

Populations of *A. euteiches* sampled in W-WA and NE-OR were similar in genotypic diversity, yet clearly differentiated with G_{ST} values between 0.3-0.4 (Table 2; Figure 2). Genetic diversity for populations in W-WA and NE-OR ranged between 2.9 and 3.7.

Table 2. Sample size (*n*), number of observed multilocus genotypes (*g*), Nei's gene diversity (*h*) (Nei 1973) and multilocus genotypic diversity (*H'*) (Grünwald et al. 2003; Shannon and Weaver 1949), and population differentiation (G_{ST}) for populations of *Aphanomyces euteiches* for two populations East and West of the Cascade Mountains, respectively.

Population	*n*	*g*	*h*	*H'*	G_{ST}
East	98	62	0.169	3.743	0.27
West	43	27	0.178	2.848	0.41

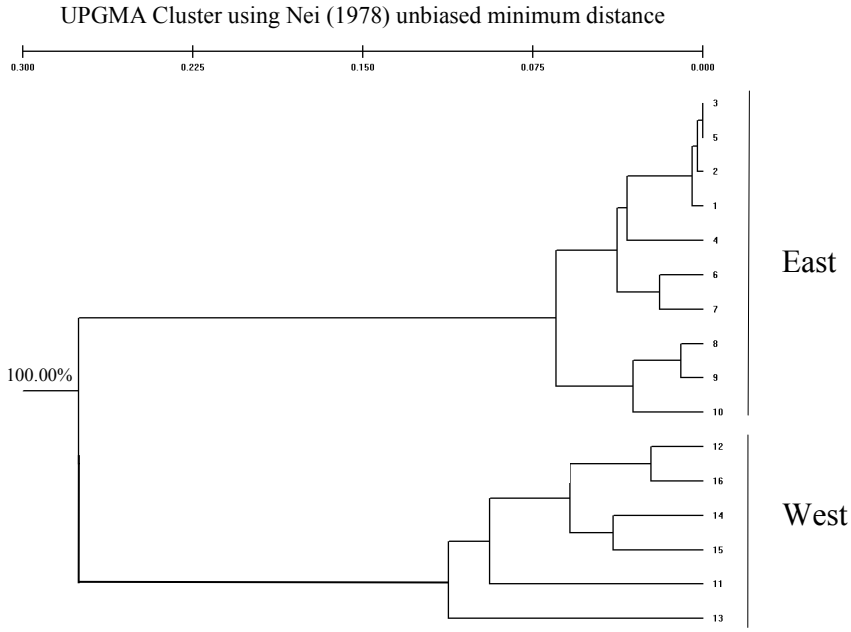

Figure 2. Cluster analysis of two populations of *Aphanomyces euteiches* East and West of the Cascade Mountains. The dendrogram was constructed using the unweighted pair-group method with arithmetic average clustering based on Nei's genetic distance (Nei 1978).

Populations of *A. euteiches* recovered from two fields are genetically diverse within a field, and are well differentiated in two geographically separated fields. At the same time, both populations are well adapted to their pea hosts and are very aggressive to the five pea lines included in this study.

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Section III: Plant Breeding

Genetic factors affecting Aphanomyces tolerance and root growth in the pea line MN313

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SUMMARY

A step-wise approach was used to identify the positions of genes responsible for the high level of tolerance to *Aphanomyces euteiches* found in the pea line MN313. A major gene on linkage group (LG) IV accounted for much of the genetic variability in an RIL population derived from a cross between MN313 and a somewhat tolerant line, OSU1026. A second gene on LG VI played a less influential role in controlling tolerance. In a second RIL population derived from the cross of MN313 with the susceptible line 'Sparkle' three genes could be distinguished: one on LG IV, one on LG VII and a minor gene on LG VI. Analysis of a third population indicated that a gene on LG VI produced a major effect on tolerance. This population was also used to establish the locations of QTLs influencing root mass and root/shoot ratio. Genes with major affects on root/shoot ratio were found on LG III and LG V. These locations did not correspond with those for the genes affecting disease tolerance, and we conclude that genetic variation in root vigor or partitioning of photosynthate is not an important factor contributing to disease tolerance.

INTRODUCTION

Common root rot, caused by *Aphanomyces euteiches*, is a serious disease of pea throughout much of the world. Previous screening of the pea germplasm has identified several sources of tolerance to the disease, and researchers such as Earl Gritton (University of Wisconsin), John Kraft (USDA), David Davis (University of Minnesota) and Rebecca McGee (General Mills) have produced commercial types with good tolerance. However, the development of such germplasm has been time-consuming due to the multigenic nature of common root rot tolerance and the strong influence of environment conditions when assaying for disease severity.

Mapping and tagging the genes conferring tolerance to common root rot has been an important objective in several recent studies (Weeden et al., 2000; Pilet et al., 2001,

2002). A region on LG VI was identified as important in the pea lines MN494, MN313 and 90-2131 (Cargnoni and Weeden, 1994). A region on LG IV also has been reported as a major gene influencing common root rot tolerance in several pea lines (Weeden et al., 2000; Pilet et al., 2002). Here we report a more thorough analysis of the segregation of tolerance to common root rot in the recombinant inbred population derived from the cross MN313 x OSU1026, as well as the results from two tolerant by susceptible crosses (MN313 x 'Sparkle' and MN313 x JI1794).

MATERIALS & METHODS

The MN313 x OSU1026 recombinant inbred population consisted of 45 F5 or F6 lines (depending on year of test) derived by single seed descent from F_2 plants. Complete data were obtained on only 42 of these lines, and the analysis is limited to this set. The plants were grown as reported in Weeden et al., 2000. The MN313 x 'Sparkle' population consisted of 93 F_2-derived RILs. This population was grown at LeSueur, MN in 2000 and 2002, and in Pullman in 2001. A third population, derived form the cross MN313 x JI 1794, consisted of 51 RILs. It was grown at Pullman in 2000 and was used for the analysis of root mass and root/shoot ratio. For all tests, plants were scored for disease severity using a scale of 1 to 5, where 1 indicates no evidence of the disease and 5 indicates a dying or dead plant. For the MN313 x OSU1026 population, this scale was converted to a three-category scale (moderately susceptible/moderately tolerant/highly tolerant) because very few lines exhibited the '4.0' to '5.0' phenotype characteristic of the truly susceptible genotypes. Linkage maps were developed for both populations using isozyme loci, sequence tagged sites, microsatellites, AFLPs and RAPD markers selected to give good coverage of the pea linkage map.

RESULTS AND DISCUSSION

MN313 x OSU1026 population

At LeSueur both parental lines exhibited tolerance to common root rot relative to susceptible controls. Only one year of data was obtained for OSU1026, but it rated significantly better than either 'Sparkle' or 'Badger,' although it did not display as high a level of tolerance as MN313. The range in tolerance exhibited by the recombinant inbred lines paralleled that shown by the parents, with only one line displaying a phenotype more susceptible than the OSU1026 parent. At Pullman, the difference between MN313 and OSU1026 was not as marked (2.5 compared to 3.5). Nor were susceptible lines significantly different from OSU1026. However, the RIL population showed a broad range of tolerance, and about a third of the lines had scores outside of the parental means.

Dividing the distributions obtained in each of the three year/locations into three classes (high, intermediate and low tolerance) revealed a good consistency among the scores a line received for the three evaluations. Over half the lines received the same score in all three evaluations and only three lines received a high tolerance score at one site and a low tolerance score at the other. The high and low tolerance groups were used to identify markers that displayed an apparent linkage with the extreme phenotypes. Two

regions of the genome were identified that showed correlation with the phenotypic segregation. One was the region on LG IV near P393 as reported by Weeden et al., (2000). The P393 allele from MN313 was associated with a significant increase in tolerance across all site/years. The second region was approximately midway between *Er1* and *Sbm1* on LG VI. The overall effect of this region (maximized at marker Pgm-F_{820}) was barely significant ($P<0.05$), but in lines possessing the OSU1026 allele at P393 gave a more definite response was observed. In this case, it was the OSU1026 allele that increased tolerance.

MN313 x 'Sparkle' population

As was true for the MN313 x OSU1026 experiments, the difference between parental lines in the MN313 x 'Sparkle' evaluations were much greater at LeSueur than at Pullman. Consistency of phenotype also was higher between the two trials performed at LeSueur than between either of these trials and that performed at Pullman. Despite some scatter of the data obtained from the two sites and from that obtained from the greenhouse inoculations, there was considerable correlation in the values obtained for common root rot tolerance for any one line. Lines consistently displaying highly tolerant or highly susceptible phenotypes were relatively easy to identify. We selected approximately 10 lines at each extreme of the distribution and were able to identify four regions on the linkage map that gave significant segregation distortion relative to extreme phenotypes.

One region with a strong effect on common root rot tolerance was located on LG VII. Mean score for the lines with the 'Sparkle' allele was 4.27, whereas that for lines with the MN313 marker was 3.30 (two-tailed t-test $P<0.0001$). The position of this gene is very close to *Sn*, the locus responsible for flowering response to daylength. In addition, segregation for early versus late flowering showed a high correspondence with this location. As 'Sparkle' is known to be *sn*/*sn*, whereas MN313 is *Sn*/*Sn*, the influence of this region on common root rot tolerance may to be due to segregation at *Sn*. In order to eliminate the effect plant maturity might have had on scoring for disease tolerance the population was divided into early and late flowering types.

Within each of the maturity groups, the region displaying the primary effect on tolerance was located on LG IV, centered on the AFLP marker E-ACT-M-AC-275. This marker is located near P393, but the precise distance between E-ACT-M-AC-275 and P393 could not be determined in this population because P393 was monomorphic. Overall, the lines with MN313 genotype had an average tolerance score of 3.59, while lines with the 'Sparkle' genotype had an average score of 4.56 (t-test $P<0.001$). This effect appears to be produced by the same gene that was identified in the MN313 x OSU1026 population. A region on LG VI, in approximately the same location as the gene identified in the MN313 x OSU1026 population, produced a minor but discernable effect on disease tolerance in certain maturity groups. In contrast to the former population, it was the allele from MN313 that increased tolerance in the MN313 x 'Sparkle' population.

MN313 x JI1794 population

The data from the population grown at Pullman, WA in 2000 showed a clear segregation of the lines into moderately susceptible (disease score about 3) and highly susceptible (score 5) phenotypes. The population was about equally divided between these two phenotypes, and the segregation pattern closely correlated with the DRR49 marker on LG VI. DRR49 codes for a pathogenesis-related protein and was isolated from pea pods after inoculation with *Fusarium solani* (Chiang and Hadwiger, 1990). This result differed markedly from those obtained for the other populations, and because JI1794 had a significantly different root/shoot ratio than MN313 (Weeden and Moffet, 2002) and its response to *F. solani* was unknown, both were examined as potential contributors to the disease tolerance phenotypes observed at Pullman. The regions affecting the root/shoot ratio in the MN313 x JI1794 population appear to be the similar to those reported in Weeden and Moffet (2002) for the JI1794 x 'Slow' population except that another major effect was observed from a region on LG V (M. Moffet and N.F. Weeden, unpublished). None of the regions identified as influencing root mass or root/shoot ratio map near DRR49 on LG VI or near P393 on LG IV, and we conclude that the genetic variation observed for root/shoot ratio is not a major mechanism for tolerance to *Aphanomyces euteiches*, at least from MN313.

The field at Pullman contained high levels of *Fusarium solani*, and this pathogen could be isolated from diseased plants grown on that field. However, comparison of JI1794 and MN313 for tolerance to *F. solani* demonstrated that JI1794 is slightly more tolerant than MN313 and that MN313 did not possess a particularly high level of tolerance (S.T. Hance, unpublished). Thus, unless there is a synergistic relationship between *A. euteiches*, *F. solani* and the allele of DRR49 present in JI1794, it does not appear that the segregation pattern in disease phenotype observed at Pullman was a result of high susceptibility of JI1794 produced by the presence of a unique DRR49 allele. We conclude that the segregation in disease phenotype observed in the MN313 x JI1794 population at Pullman was a result of a unique aspect of the JI1794 genotype on LG VI that may or may not involve DRR49.

OVERALL CONCLUSIONS

Our results indicate that three regions of the MN313 genome contribute to common root rot tolerance in the field. The region that appears to be most important in a cross to a susceptible line ('Sparkle') is on LG VII very near *Sn*. It is known that scoring for disease tolerance may be biased by differences in maturity, with early maturing genotypes appearing more susceptible because of their generally shorter stature and earlier senescence (J. Kraft, pers. commun.). We attempted to circumvent this complication by examining root symptoms as well as above ground appearance; however, it is possible that the influence we observed on LG VII is merely a pleiotropic effect of the *sn* gene and does not truly reflect a difference in susceptibility. However, if the LG VII influence is bogus, we are left without an explanation for the difference in susceptibility observed between OSU1026 and 'Sparkle.'

The region on LG IV produced a major effect in the two populations most intensely investigated and appears to be an important gene in MN313 contributing to tolerance to common root rot. Its position is similar to *Aph1* described in Pilet et al. (2002). If MN313 does contain *Aph1*, it suggests that this gene was introduced into the tolerant material from both the Western and Midwestern breeding programs in the United States. The presence of this gene in the two main sources of resistance to common root rot implies that relatively few tolerance genes are available in the *Pisum* germplasm. Genes with more subtle effects, such as that on LG VI in OSU1026 and those described in Pilet et al. (2002) thus become very important to further characterize.

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Genetics of partial resistance to Aphanomyces root rot in pea

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SUMMARY

In order to better understand genetics of quantitative resistance to *Aphanomyces euteiches* in pea, this study aimed to study the consistency of 3 resistance QTL previously identified (*Aph1*, *Aph2*, and *Aph3*; Pilet-Nayel *et al*., 2002) by comparative mapping in another genetic background. A mapping population of 111 RILs, from the cross DSP (susceptible) x 90-2131 (partially resistant), was assessed for field Aphanomyces root rot resistance over 6 environments (2 US and 2 French locations, 1 to 3 years per location). A partial genetic map, consisting in 30 linked markers and covering the genomic regions including *Aph1*, *Aph2* and *Aph3*, was elaborated from the RILs. A total of 6 QTL were identified from only 3 environments (2 US and 1 FR) and explained individually up to 12% of the variation. One QTL (*QTL1*), identified from Pullman 2000 data, co-localized with *Aph1* but the others did not co-segregate with *Aph1*, *Aph2* or *Aph3*. The hypothesis of the involvement in the resistance of other genetic factors in other regions of the genome is highlighted and is under investigation.

INTRODUCTION

Aphanomyces root rot Drechs., due to the fungus *Aphanomyces euteiches*, is one of the most important diseases in many pea-growing areas (North America, Australia, New Zealand, Japan and Europe, especially in France). No efficient chemicals are currently available to control the pathogen. The use of resistant varieties, associated with cultural and prophylactic methods of control, appears to be a promising strategy to control the disease. Pea breeding for resistant varieties has been difficult, particularly due

to the polygenic inheritance of resistance/tolerance. In order to assist pea breeding efforts for resistance and better understand genetics of quantitative resistance to *A. euteiches* in pea, one of our objectives is to identify QTL associated with Aphanomyces root rot partial resistance and analyze their consistency towards environment, genetic background and pathogen variability. In a previous study, we identified 7 genomic regions associated with the resistance in the cross 'Puget' (susceptible) x 90-2079 (partially resistant), among which *Aph1*, a "major" QTL on linkage group IV (LG IV), *Aph2* on LG V and *Aph3* on LG I, two other consistently revealed QTL (Pilet-Nayel *et al*., 2002). In the present study, our objective was to study the consistency of these 3 QTL in another cross involving another partial resistance germplasm, 90-2131 (Kraft, 1992) over US and French environments especially differing for their pathogen populations (Wicker and Rouxel, 2001).

MATERIALS & METHODS

A population of 111 F_{10}-derived Recombinant Inbred Lines (RILs), from the cross DSP (susceptible) x 90-2131 (partially resistant) was produced at USDA Pullman (WA, USA) and used for QTL analysis.

The lines were assessed for field partial resistance to *A. euteiches* at 4 locations (Pullman, WA, US; LeSueur, MN, US; Riec-sur-Belon (29), FR; Dijon (21), FR) and in 1 to 3 years (Table 1), using a randomized complete block design with three replications. Two scoring criteria based on symptom severity and disease effects were used to evaluate the lines (Table 1) : i)- the root rot index (RRI) on 10 plants per plot at the 5-6 leaf stage and ii)- the above ground index (AGI) on each whole plot from the flowering stage. Variance analysis of field data for each resistance criterion in each environment were performed using the SAS program.

The RILs were genotyped using SSRs (Agrogène Company, Moissy-Cramayel, France) and RAPDs (Opèron Technologies, Alameda, CA, USA) markers. A partial genetic map was elaborated with the Mapmaker/Exp v3.0 software. QTL mapping analysis was conducted by Composite Interval Mapping (CIM), using the program QTL-CARTOGRAPHER v1.3 for MS-Windows.

Table 1: Environments (locations, years) in which (DSP x 90-2131) RILs were evaluated for resistance criteria to *A. euteiches*

Location	2000	2001	2002
Riec-sur Belon (FR)			RRI
Dijon (FR)			AGI (2 dates)
LeSeur (US)	AGI	AGI (2 dates)	AGI (2 dates)
Pullman (US)	AGI		

RRI : Root Rot Index; AGI : Above Ground Index

RESULTS & DISCUSSION

Gaussian frequency distributions of the scoring criteria on the RILs were observed in each environment, suggesting the polygenic nature of the resistance. A partial genetic map, covering the genomic regions including *Aph1* (LG IV), *Aph2* (LG V) and *Aph3* (LG I) (Pilet-Nayel et al., 2002), was elaborated from 30 markers (2 morphological markers, 11 SSRs and 17 RAPDs), with 26 markers mapped on previous genetic maps (Figure 1).
A total of 6 QTL were identified for Aphanomyces resistance in 3 of the 6 environments tested (Table 2). No QTL was detected from LeSueur 2000, 2001 and Riec-sur-Belon 2002. All the QTL identified explained individually a low part of the variation (<12%). Resistance alleles at four of them derived from the susceptible parent (DSP). None of the QTL revealed in the US and FR environments co-segregated.
Two QTL (*QTL1* and *QTL2*), obtained from US data, were localized in the same genomic region as *Aph1* (Figure 1). However, the resistance allele at *QTL2* did not derive from the resistant parent as for *QTL1* and *Aph1*, suggesting that *QTL2* would correspond to a different locus. The other four QTL did not co-segregate with *Aph1*, *Aph2* or *Aph3*.
These results show the poor consistency of *Aph1*, *Aph2* and *Aph3* over the 2 genetic backgrounds tested, suggesting that i)- either there is no allelic polymorphism at these 3 QTL in DSP x 90-2131 or ii)- other genetic factors localized on other genomic regions are associated with the resistance in 90-2131, which would make sense regarding the different genealogies of the two partially resistant parents (90-2079 and 90-2131, Kraft, 1992)).

Work is in progress to study QTL consistency over the two genetic backgrounds on the whole genome including 2003 field and controlled conditions data.

Table 2: Statistical parameters associated with QTL detected for field Aphanomyces resistance in the (DSP x 90-2131) RILs population (values obtained by QTL-Cartographer v1.30/Win)

Year	Location	Scoring criterion [a]	QTL	Linkage group	LOD [b]	Additive effect [c]	R^2 [d]
2000	Pullman (US)	AGI	QTL1	IV	2.38	0.54	0.12
2002	LeSueur (US)	AGI11st	QTL2	IV	2.20	-0.19	0.08
2002	Dijon-Epoisses	AGI-1st	QTL3	IV	2.83	-0.23	0.09
			QTL4	V	3.50	0.25	0.11
2002	Dijon-Epoisses	AGI-2nd	QTL5	IV	2.80	-0.24	0.09
			QTL6	VI	3.57	-0.25	0.12

[a] AGI : Above Ground Index, [b] Peak of Logarithm of Odds, a LOD threshold of 2.2 was defined using the permutation test of the software; [c] Effect of substituting '90-2131' alleles for 'DSP' alleles at the LOD peak of the QTL; [d] % variation explained by an individual QTL.

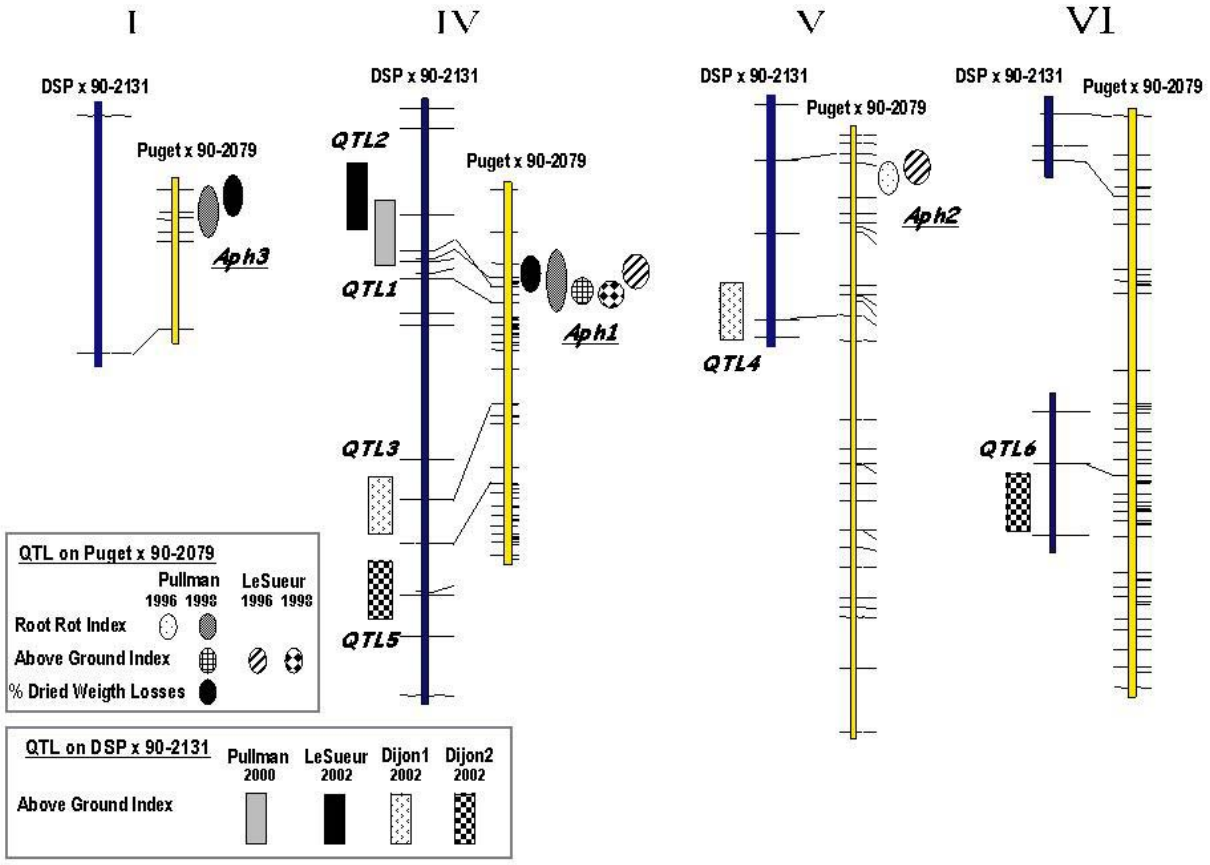

Figure 1 : Genomic localization of QTL for resistance to *A. euteiches* detected in the cross "DSPx90-2131". Comparison with localization of 3 QTL for resistance to *A. euteiches* in the cross "Puget x 90-2079" (Pilet-Nayel *et al*. 2002)

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Application of SNPs in identifying new positive alleles for genetic resistance to Aphanomyces root rot in pea germplasm

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SUMMARY

Allele mining in pea germplasm will allow the genetic resources user community to directly access new useful genetic variation for genetic resistance to Aphanomyces root rot in pea. The development and refinement of co-dominant markers for Aphanomyces and Fusarium root rot resistance in pea is underway through cooperative research projects (Inglis, McPhee, Grunwald, McGee, Pilet-Nayel) to complete the multiple environment phenotyping necessary and genotyping for QTL discovery in recombinant inbred populations. The QTLs are under study in two RIL populations: Puget x 90-2079 (Aphanomyces) and Dark Skin Perfection x 90-2131 (Aphanomyces and Fusarium). Three QTL for tolerance to Aphanomyces in pea have been identified in multiple field environments and under greenhouse conditions. SNP identification of these loci is underway.

INTRODUCTION

One of the major constraints to fully utilizing the U.S. plant germplasm resources is the absence of efficient methods to discover new alleles for use in crop breeding programs (Tanksley and McCouch, 1997). The application of genomics to plant model species Arabidopsis (The Arabidopsis Information Resource http://arabidopsis.org/) and especially from model legume species *Lotus japonicus* (TIGR Lotus japonicus Gene Index http://www.tigr.org/tdb/tgi/ljgi/) and *Medicago truncatula* (Medicago truncatula Consortium http://www.medicago.org/) are delineating the pathways and specific genes associated with economic traits. Saturated pea maps and phenotyping of RILs led to discovery of many useful markers in pea (Rameau, Ellis, Timmerman-Vaughan, Weeden). Particularly, robust QTLs for tolerance to Aphanomyces root rot of pea were identified on a medium density linkage map of Puget X 90-2079 (Pilet-Nayel *et al*. 2002).

How can these markers be applied to answering the plant breeding dilemma of efficient utilization of new positive alleles in germplasm collections without disrupting positive linkage blocks assembled over decades by generations of pea breeders?

SNPs have been shown to be useful for allele discovery and utilization in maize (Remington *et al* 2001). Single nucleotide polymorphisms are the most common type of sequence difference between alleles (Rafalski, 2002), including pea (Table 1). Further, there is potential for the use of SNP haplotypes in the detection of associations between allelic forms of a gene and phenotypes (Rafalski, 2002), especially quantitative disease resistances like Aphanomyces resistance.

The limitations of SNP analysis vs. standard marker development are that sequence knowledge is required, technology is still needed for a standard plant genetic analysis laboratory, and determination of which SNP/INDEL is responsible for the + phenotype has been very difficult until recently (Rafalski, 2002; Buckler and Thornsberry, 2002).

MATERIALS & METHODS

Alleles of pea genes that are currently available through cooperators include twelve pea genes from Gail Timmerman-Vaughan (Table 1) and 110 pea genes from Douglas Cook (personal communication).

Table 1. Twelve pea genes with the alleles from contrasting pea lines sequenced and base pair compared to identify SNPs and INDELs.

Sequence ID	Genotypes	SNP(s)[1]	INDEL(s)[2]	Allele base pairs
P446	4	1[3]	-	-
E09_704	2	24	3	701
F1C	2	22	6	2170
InvP3P2d	2	3	1	525
K05_1005	2	43	4	1032
M02_1230	2	16	2	1231
O12_518	2	18	4	579
P2P2c	2	6	0	200
P2P2d	2	2	3	756
P2P5j	2	1	0	940
P3P2c	2	3	1	287
PyrB2	2	10	2	440

[1] SNP = single nucleotide polymorphism.
[2] INDEL = insertion or deletion of multiple nucleotides.
[3] One SNP assay completed for this gene.

SNPs linked to Aphanomyces root rot resistant QTL in pea will be developed using procedures reviewed in Rafalski (2002). The seven loci under SNP development from Pilet-Nayel et al. 2002 are listed in Table 2. The three QTL (*Aph1*, *Aph2*, and *Aph3*) have been confirmed in greenhouse tests (Coyne and Pilet-Nayel, unpublished data). Each marker will be cloned and sequenced in both directions. Alleles will determined in the four RIL parents and in the pea BAC library genotype, PI 259818. Alleles will be aligned using ClustalX (hgmp.mrc.ac.uk/Registered/Option/clustalx.html) to identify SNPs and any INDELs. SNP assays will be developed using two approaches.

The first was the Promega READIT SNP kit which identifies SNP polymorphism with a simple but costly luminometer assay. The second approach utilizes a capillary electrophoresis unit for the SNP genotyping (Beckman Coulter CEQ 8000). Both require the same PCR conditions and primers without a PCR reaction cleanup step, with the main difference being in the detection label. Both assays benefit from use of a liquid handling robot (Qiagen BioRobot 3000).

Table 2. SNP assay will be developed for the seven QTLs detected for field Apahnomyces resistance in pea (Pilet-Nayel *et al*. 2002).

QTL name	Marker[1]	Maximum LOD	R^2
Aph1	E7M4.251	20.4	0.47
Aph2	E7M2.254	10.1	0.32
Aph3	U370.900	4.0	0.11
Aph4	E2M4.249	3.2	0.06
Aph5	E3M3.167	6.5	0.13
Aph6	Pgdp	3.7	0.06
Aph7	E1M4.174	4.2	0.07

[1] Marker detail published in Pilet-Nayel *et al*. 2002.

RESULTS & DISCUSSION

In order for the SNP application to USDA pea germplasm collection to be useful for identifying positive alleles for Aphanomyces root rot resistance, which polymorphism responsible for phenotype must be determined. Association genetics has proven successful in identifying the polymorphism responsible for a maize phenotype (Remington *et al*. 2001; Allele Mining Workshop http://www.intl-pag.org/11/abstracts/W02_W10_XI.html) and for allele discovery in the CIMMYT maize germplasm collection. Buckler and Thornsberry (2002) outline the steps necessary for association mapping in germplasm collections. The first step is to determine the population structure using molecular diversity analysis, second collect phenotypes, and third run association test statistic with an incorporated estimate of population structure.

The USDA pea germplasm core collection population structure will be determined using mapped RAPDs (Laucou *et al*. 1998) and published SSRs (Burstin *et al*. 2001) for pea. As markers linked to Aphanomyces resistance QTL are sequenced, GenBank searches are conducted to see if any registered accessions are homologous, particularly in the *Medicago truncatula* EST and BAC sequencing databases (http://www.medicago.org/). SNP assays will be conducted on the pea core collection of 360 accessions, refined from the original geographic core of 505 accessions. A min-core of 50 accessions will be used to first test each SNP assay. The mini-pea core selection was based on data from biomass (McPhee and Muehlbauer, 2001) and mineral nutrition (Michael Gruzak, unpublished data) studies to create a correlation matrix. The matrix was used for cluster analysis (UPGMA) using the NTSYSpc software.

Potentially the gel-free SNP assays developed will have higher through-put than other gel-based marker systems. The result may be molecular selection of positive new

alleles for Aphanomyces root rot resistance from the pea germplasm collection without losing the positive linkage blocks in current elite cultivars and breeding lines of pea.

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Challenges in breeding grain legumes for resistance to fungal pathogens

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SUMMARY

Fungal pathogens of grain legumes cause significant crop damage resulting in millions of dollars in lost revenue and have resulted in some crop land being taken out of production due to presence of exceptionally high inoculum levels. Resistance breeding has faced numerous challenges, but has been successful in developing improved germplasm especially with regard to disease resistance controlled by single genes and somewhat less successful with those resistance traits controlled by multiple genes or those that are highly responsive to environmental factors. Some of the challenges encountered during the stages of resistance breeding will be discussed as well as some of the perceived needs and potential challenges which will be faced in the future. Overall, disease resistance breeding is becoming increasingly more complex through greater understanding of the host-pathogen interaction and introduction of more sophisticated technology. Successful deployment of new and more modern approaches to resistance breeding will require a multidisciplinary approach as well as greater investment in resources such as equipment, time and labor.

INTRODUCTION

Grain legumes are host to many pathogens that cause disease. These diseases result in millions of dollars lost in crop production, reduced seed quality and pesticide applications. Two examples are Ascochyta blight of chickpea caused by *Ascochyta rabiei* and powdery mildew of pea caused by *Erysiphe pisi*. Ascochyta blight has a devastating impact on crop production while powdery mildew reduces crop production, seed quality and heavy spore production can have adverse human health effects as well as result in harvesters catching on fire in the field. Additionally, disease incidence caused by high inoculum levels in some areas has resulted in removal of legume crops from rotations. An example in the eastern Palouse region of northern Idaho is pea which is no longer grown in high rainfall areas due to soils infested with *Aphanomyces eutieches* and other pathogens contributing to the root rot complex.

Breeding for resistance to many pathogens affecting grain legumes has been rather successful; however, breeding for resistance to some pathogens, especially soilborne fungal pathogens has been very difficult. Challenges to breeding for resistance to fungal pathogens is primarily associated with those controlled by multiple genes and quantitative trait loci (QTL). Disease resistance controlled by a single or few genes has

been readily incorporated into most cultivars currently in production. In fact, resistance controlled by single genes, particularly in pea, have been remarkably durable for many years, i.e. *er* for resistance to powdery mildew and the dominant genes, *Fw, Fnw,* and *Fwf*, for resistance to Fusarium wilt.

Disease resistance breeding has become increasingly complex over time due to inherent difficulties associated with disease evaluations coupled with the vast amount of information that is being generated on the pathogen and host genomes as well as the many new technologies that are being used to identify resistance genes. Despite the advances that are being made on host-pathogen resistance, there is still a large void in understanding the sophisticated mechanisms pathogens use to colonize their hosts and the response mechanisms used by the host to resist infection and prevent disease development.

This presentation will attempt to outline a generalized sequence of events followed to develop disease resistance and some of their associated challenges. The initial stage of resistance breeding can be simply viewed as identifying of the pathogen. Identification of the pathogen is limited by the multiplicity of pathogens that colonize damaged tissue secondarily making it difficult to identify the causal organism initially. The second stage includes studies of disease epidemiology and development of cultural practices to eliminate or reduce disease incidence and impact. Development of cultural practices is hampered by time consuming experimentation and interpretation of anecdotal observation. However, once appropriate cultural practices are identified and implemented they are highly effective in reducing the economic impact of disease.

Parallel to pathogen identification and development of cultural control practices a third stage begins with identification of resistant germplasm and determination of the genetics of resistance. These investigations are commonly limited by establishment of suitable screening methodology either under field conditions or in controlled environments and development of accurate scales to score disease symptoms. Several scales have been developed for various diseases, but many suffer from subjectivity based on individual interpretation. Field trials can be very difficult to interpret and are variable from year to year due to differences in inoculum and various weather components. Therefore the repeatability of field trials is often difficult and extends the time required to develop resistant germplasm.

Due to these difficulties in the field and the emergence of DNA technologies, the fourth stage of disease development begins with identifying genomic regions responsible for resistance and tagging them with molecular markers that could be used to identify germplasm with a high likelihood of containing the specific resistance gene. As more sophisticated methodology was developed additional tests became available to more accurately predict the presence of resistance genes. Currently, the molecular markers which have been identified and tag specific resistance genes represent a diversity of techniques. Breeding programs handling thousands of individual selections and breeding lines are often not equipped to apply a large number of different techniques. Therefore,

there is a need to develop transportable molecular markers based on one or few techniques.

As more detailed understanding of the genetics controlling the host-pathogen interaction becomes available, it has become possible to investigate mechanisms of resistance and pathogenicity at the molecular and biochemical level. This detailed knowledge will make more precise approaches to developing disease resistance available and will lead to a fifth stage of resistance breeding involving the manipulation of resistance genes in an attempt to control disease development and maximize durability of resistance. This step is still on the horizon and requires a significant amount of additional information on the process of disease development and mechanisms of resistance before successful deployment of precision resistance can be realized. Although it will be a powerful approach, it will add substantial complexity to the breeding process and require significant changes in the approach to resistance breeding.

In summary, understanding disease development and resistance mechanisms has become increasingly more detailed and complicated with time. Although knowledge within the research community is expanding, the increased amount of information and the highly technical nature of the molecular techniques will require substantial investment in resources such as equipment, time and labor to successfully integrate these new advancements into breeding programs. Implementation of new technology and approaches to disease resistant breeding will continue to be a significant challenge in the future.

Plant breeding requires a multidisciplinary approach including plant pathology to successfully develop improved germplasm or cultivars. Similar to plant breeding the plant pathology component should also be viewed as a multidisciplinary endeavor requiring a team approach involving many of the same areas of expertise. Successful integration of these areas will provide the greatest opportunity to advance the state of the art and minimize the impact of current and future challenges facing disease resistance breeding.

GSP Breeding program for resistance to Aphanomyces root rot

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The objective of the program is to provide to the breeders, members of the GSP, parent lines with a good level of resistance against aphanomyces, and with a good agronomical value.

GSP (Groupement des Sélectionneurs de Pois protéaginux) is composed of seven breeding companies having their pea research program in France : GAE Recherche, SERASEM, F.Desprez, UNISIGMA, Cebeco Semences , Momont and Nickerson. A Technical team of three people leading by Martine Roux-Duparque is hosted at the INRA Station of Mons in Picardie where the GSP program is organised. GSP is largely supported by UNIP, the French integrated chain association involved in Grain Legumes, from the production toward the end uses (feed, food). The main activity of GSP is to transfer the research results coming from the public institut (INRA) directly to the breeders for their own breeding programs.

The GSP Aphanomyces program have started in 1994/1995, in collaboration with extension services who had to reply urgently to the strong demand from the farmers. A first crossing programme started in 1995 with some american lines coming from Kraft and Gritton. In collaboration with the Pathology team of INRA Rennes, a method of test in controlled conditions was set in 1999, and is used now as a first screening of the genetic material prior to the field assessment. In 2001, a financial support coming from the French Ministry of Agriculture have boost the GSP programme and gave the opportunity to create a field network of 8 locations that allows to evaluate and confirm the value of the material coming from the several crossing programs. For 6 of these locations, a replicate of the trials is performed in a non infested soils closed to the infested one, to allow a good comparison between the yield potential in the location and the yield losses of the lines due to aphanomyces.

The last results show that it is possible to introduce the resistance coming from the American lines into the European cultivars, but the level of resistance is still low to solve the problem at the farm level, and the search of new sources of resistance is required; the screening of genetic resources done by INRA will.provide hopfully some new material to work with .

Moreover, the field trials network point out the heterogeneity of the root rot complex, including fusarium solani and fusarium oxysporum, as well as phoma medicaginis. The assessment of the resistance of the material against aphanomyces can be affected by the other diseases in a same field and the field network set up by the GSP ensure each year to the breeders a good result in some few locations among the 8 ones.

Other appleid research in France are focusing on the cropping control of the disease and concerns predictive test of the disease in the field, cover crop effect on the impact of the disease, seed treatment, and soil structure and pathogen interaction. All of these programs are taken into account in the breeding programs.

Association mapping to identify marker loci associated with resistance to Aphanomyces root rot of pea in New Zealand

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SUMMARY

Research is underway to identify pea lines resistant to *Aphanomyces euteiches* root rot of pea as it occurs in New Zealand, to understand the genetics of resistance and to identify molecular markers linked to resistance loci. One approach being taken is to genotypically characterize a group of 134 breeding lines that vary for resistance and to use those data to conduct tests of association between trait values and marker or candidate gene alleles. Association tests are being conducted following an approach that first estimates the population structure of the lines based on their molecular marker genotypes, then tests the association between the trait value for the lines versus marker genotypes, taking into consideration the putative subpopulation. Research to date has demonstrated that the collection of breeding lines is likely to have a population structure based on allelic variation at 42 AFLP markers. Results for a predicted 3 subpopulations are presented. Aphanomyces root rot disease scores in the New Zealand environment are being determined in trials in fields that have a high soil index. Before association tests are conducted, further research will increase the number of polymorphic molecular markers and include candidate genes as well as molecular markers previously shown to be linked to resistance QTLs.

INTRODUCTION

Aphanomyces root rot of pea, caused by *Aphanomyces euteiches*, has become a significant problem in New Zealand, and contributes to reduced pea production, particularly of field pea genotypes. Aphanomyces root rot was first detected in New Zealand in 1977, and was followed by reports in 1979 and 1980. Major pea growing regions are affected, including Nelson, Canterbury, Hawkes Bay and the Horowhenua. While the economic impact has not been assessed, attempts to grow peas on infested soils can lead to complete crop loss. Avoidance is currently the only available control measure. Growers use soil testing to assess the degree of infestation, and long rotations to minimize the build up of the pathogen in soils.

Association mapping, using the case-control study method, is widely used in human genetic research for identifying candidate gene alleles or tightly linked marker

alleles that are associated with a trait of interest (reviewed in Pritchard et al. 2000b). A serious limitation of case-control studies is the risk of obtaining false associations if the populations of individuals making up the study groups have an underlying structure. Case control studies have not been used in crop plants, because most crop plant germplasm does have underlying population structure as a result of domestication and selection processes. More recently, methods have been developed to conduct case control studies in the presence of population structure (Pritchard et al. 2000b). The software developed by Pritchard et al. (2000a) uses a Bayesian approach to characterize population structure based on individuals' genotypes at unlinked loci using molecular markers, then conducts association tests, taking into account the estimated population structure. Recently this approach has been used in *Zea mays* to explore the association of *Dwarf8* polymorphisms with flowering time (Thornsberry et al. 2002).

We have initiated a research program aimed at developing pea varieties that are resistant to Aphanomyces root rot in New Zealand. Our aim is to characterize the genetics of resistance, identify linked molecular markers, and use marker-assisted selection to develop resistant cultivars. Using the statistical approach developed by Pritchard et al. (2000), we have characterized the population structure of pea breeding lines that vary for resistance to Aphanomyces root rot, as a first step toward identifying candidate genes or tightly linked markers associated with resistance genes.

MATERIALS & METHODS

A collection of 134 pea breeding lines and cultivars was genotyped with AFLPs (amplified fragment length polymorphisms) using the DNA marker methods described by Timmerman-Vaughan et al. (2002). Four primer combinations were used: MseP2/PstP2 (Mse+GAA/Pst+GT), MseP2/PstP3 (Mse+GAA/Pst+GT), MseP3/PstP2 (Mse+GAG/Pst+GT) and MseP5/PstP2 (Mse+GAT/Pst+GT). Forty three polymorphic bands were scored.

Population structure was estimated using the software STRUCTURE 2.0 with the Windows front-end (Pritchard and Wen 2002). Population structure simulations were run iteratively for population number (k) values from 1 through 5, with a Burnin period of 100,000, followed by 100,000 MCMC (Markov chain Monte Carlo) replications. The proportion of each individual line's genome originating in the putative subpopulation (q) was estimated. Two models were tested: with admixture, and with no admixture.

RESULTS & DISCUSSION

The 134 breeding lines selected for population structure estimation include 123 lines developed by John Kraft and characterized for resistance to Aphanomyces root rot (John Kraft, personal communication). The remaining 11 lines are a mixture of breeding lines from the Crop & Food Research field pea breeding programme and lines that have shown resistance to Aphanomyces root rot in some environments (eg. MN313).

Figure 1. Estimation of population structure for *k*=3 putative subpopulations using the admixture and no admixture models. Bar graphs plot individual lines (x-axis) versus *q* (the proportion of each individual's genome that originated in the putative subpopulation, y-axis). Mid grey = subpopulation 1, light grey = subpopulation 2, dark grey = subpopulation 3. Panels A and B: assignment of individual pea breeding lines to one of three putative subpopulations, Panels C and D: visualization of population structure, individual lines sorted by *q*.

A: lines in order, no admixture model

individual

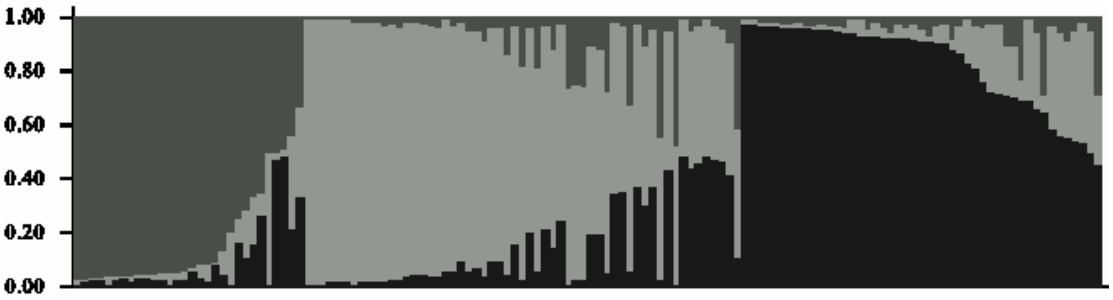
B: lines in order, admixture model

individual

C: lines sorted by q, no admixture model

individual

D: lines sorted by q, admixture model



As a first step toward using an association mapping approach for identifying molecular markers and candidate genes associated with resistance to Aphanomyces root rot, the putative structure of this collection of pea breeding lines was analyzed using the software STRUCTURE and genotype data for 42 AFLP markers. The solution for a putative population number (*k*) of 3 is shown in Figure 1. Two models were used for estimating the population structure: the admixture model (individuals draw some fraction of their genome from each of the *K* populations), and the no admixture model (each individual's genome comes purely from one of the *K* populations). Panels A and B of Figure 1 compare the proportion of each individuals genome (*q*) that is assigned to each of the three putative populations under the admixture and no admixtures models. For most individuals, the assignment to a putative population is the same, regardless of the model. Panels C and D of Figure 1 show the population structure (individuals have been sorted on *q*).

Pritchard and Wren (2002) suggest that the admixture model is the best starting place for population structure estimation since it is flexible and can accommodate complexities found in real populations. Since most of the lines are derived from John Kraft's breeding program, some shared ancestry is expected, therefore the admixture model is likely to be most appropriate. However, the no admixture model was also run for comparison.

Three sets of Aphanomyces root rot resistance scores are available for these lines: *Aphanomyces euteiches* root rot ratings based on pure culture screening in the greenhouse (John Kraft, personal communication), and above ground index and root rot index scores taken in two field trials conducted in New Zealand during the 2001-02 and 2002-03 Southern summers.

Before association tests can be conducted, further work is needed. We aim to increase the number of polymorphic molecular markers scored in this population of breeding lines, and also to determine the allelic variation for markers linked to QTLs that have been identified by Pilet-Nayel et al. (2002) and for candidate gene loci.

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