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Use of real-time PCR to examine the relationship between disease severity in pea and *Aphanomyces euteiches* DNA content in roots

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Abstract

Aphanomyces euteiches causes severe root rot of peas. Resistance is limited in commercial pea cultivars. Real-time fluorescent PCR assay specific for *A. euteiches* was used to study the relationship between disease severity and pathogen DNA content in infected peas. Five pea genotypes ranging in levels of resistance were inoculated with five isolates of *A. euteiches*. Plants were visually rated for disease development and the amount of pathogen DNA in roots was determined using the PCR assay. The susceptible genotypes Genie, DSP and Bolero tended to have significantly more disease and more pathogen DNA than the resistant genotypes 90-2079 and PI 180693. PI 180693 consistently had less disease, while 90-2079 had the lowest amount of pathogen DNA. The Spearman correlation between pathogen DNA quantity and disease development was positive and significant ($P < 0.05$) for three isolates, but was not significant for two other isolates. This suggests that the real-time PCR assay may have limited application as a selection tool for resistance in pea to *A. euteiches*. Its utility as a selection tool would be dependent on the correlation between disease development and pathogen DNA content for a given pathogen isolate. The accuracy and specificity of the real-time PCR assay suggests considerable application for the assay in the study of mechanisms of disease resistance and the study of microbial population dynamics in plants.

Introduction

Root rot of pea (*Pisum sativum*) caused by *Aphanomyces euteiches* has been observed in many regions throughout the world where peas are grown, including northern Europe, Japan, Australia and New Zealand (Pfender, 2001). Recently, considerable losses in pea production were observed in France due to Aphanomyces root rot (Wicker et al., 2001). Entire pea crops were destroyed in severely infested fields when mild and wet conditions were prevalent during seedling emergence (Pfender, 2001).

Currently no commercially-available pea cultivars have been developed with high levels of resistance to *A. euteiches*. The majority of pea breeding programmes have used an integer scale to score severity of disease caused by *A. euteiches*. Scales have been used that range from 1 to 10 (Shehata et al., 1976; Gritton, 1990), 1 to 6 (Shehata et al., 1983), 1 to 5 (Rao et al., 1995; Pilet-Nayel et al., 2002), or 0 to 5 (Davis et al., 1995; Malvick and Percich, 1999), in which disease severity is rated in ascending order from no visible symptoms to a dead plant. The primary constraint in the use of the rating scale is that subtle differences in resistance may be indistinguishable based on a visual assessment of disease severity.

Several factors, including the nature of the inheritance of resistance, are likely to be responsible for the limited gains in pea improvement. Seven quantitative trait loci (QTL) conditioning resistance to *A. euteiches* have been identified, but only one, *Aph 1*, was significantly associated with resistance over multiple years, locations, and selection criteria (Pilet-Nayel et al., 2002). Heritability estimates

(Nyquist, 1991) for resistance in peas to *A. euteiches* based on severity of root rot symptoms have not exceeded 0.50 (Shehata et al., 1983; Pilet-Nayel et al., 2002), providing additional evidence that environmental variance may contribute considerably to total phenotypic variance. Linkage between resistance genes and loci responsible for undesirable production traits, such as tall plants (*Le*), colored flowers (*A*), and colored seed (*Pl*), has also impeded resistance breeding (Marx et al., 1972). However, in recent years breeders have been able to break deleterious linkages and have released several germplasm sources that have both desirable production characteristics and at least partial resistance to *A. euteiches* (Davis et al., 1995; Gritton, 1990; Kraft and Coffman, 2000).

Several investigations have attempted to quantify both initial infection events and subsequent pathogen colonization associated with the infection of peas by *A. euteiches*. Microscopy has proven effective at studying chemotaxis of *A. euteiches* zoospores (Cunningham and Hagedorn, 1962) and zoospore germination in response to pea root exudates (Kraft and Boge, 1996). Other groups have used different methods to quantify the amount of *A. euteiches* in infected roots. Quantification methods have included counting oospores in excised root tips (Morrison et al., 1971), using polyclonal antiserum (Kraft and Boge, 1994), fatty acid profile analysis of infected plants (Larsen et al., 2000; Larsen and Bødker, 2001), and measuring the activity of fungal alkaline phosphatase and an *A. euteiches*-specific glucose-6-phosphate isozyme in infected roots and root extracts (Kjøller and Rosendahl, 1998). These techniques have been used to examine the relationship between disease severity and pathogen content in resistant and susceptible plants (Morrison et al., 1971; Kraft and Boge, 1994), oospore development during the disease cycle (Kraft and Boge, 1996; Kjøller and Rosendahl, 1998; Larsen et al., 2000) and interactions between *A. euteiches* and arbuscular mycorrhiza (Bødker et al., 2002; Larsen and Bodker, 2001). However, some of these methods have limited specificity toward *A. euteiches*. For example, several of the fatty acids examined, including 20:4 and 20:5 are common to other oomycetes and arbuscular mycorrhizal fungi (Larsen et al., 2000). The polyclonal antiserum was found to cross-react with *Pythium ultimum* (Kraft and Boge, 1994).

Techniques available for real-time detection of fluorescent-labeled PCR products (amplicons) may provide a method for accurately and specifically quantifying *A. euteiches*. Real-time PCR assays use the 5′ nuclease activity of *Taq* polymerase to generate fluorescence in proportion to the number of target amplicons produced (Holland et al., 1991). Recently, Vandemark et al. (2002) developed a real-time PCR assay specific for *A. euteiches*. In alfalfa, positive and significant correlations were observed between the severity of disease and the amount of pathogen DNA detected with the real-time PCR assay (Vandemark et al., 2002; Vandemark and Barker, 2003). In peas, significantly less *A. euteiches* DNA was detected in roots of the moderately resistant pea germplasm 90-2079 (Kraft, 1992) than in the roots of the susceptible cultivar Bolero (Vandemark and Barker, 2003). The Spearman rank correlation between pathogen DNA content in pea roots and disease severity index (DSI) ratings was positive ($\rho = 0.57$) and highly significant ($P < 0.0001$).

This assay has been proposed as a tool for selecting alfalfa with high levels of resistance to *A. euteiches* (Vandemark et al., 2002). However, unlike alfalfa, for which four isolates of *A. euteiches*, two each of two physiological races, were examined (Vandemark et al., 2002; Vandemark and Barker, 2003), only a single isolate of *A. euteiches* was tested in experiments with peas. Considerable variation in pathogenicity was observed among isolates of *A. eutieches* that infect pea (Malvick et al., 1998; Malvick and Percich, 1999; Wicker and Rouxel, 2001; Wicker et al., 2001). Most recently, Wicker and Rouxel (2001) used a set of six differential pea genotypes to identify 11 virulence types among 108 isolates of *A. euteiches* isolated from pea. This variation may adversely affect the durability of resistance in different environments. It would be prudent to examine other isolates of *A. euteiches* to determine if the real-time PCR assay is useful as a selection tool in peas.

Our objective was to use the real-time PCR assay to examine, using five isolates of *A. euteiches*, the relationship between disease severity and the amount of pathogen DNA detected in roots of five pea lines known to have different levels of resistance to *A. euteiches*. Applications of this assay for breeding programs, research on disease

resistance and the study of microbial population dynamics are considered.

Materials and methods

Pathogen isolates and plant materials

Five isolates of *A. euteiches* obtained from field-grown peas were used: C11, F3, D1, E10 and B3. Isolates C11, F3, D1 and E10 were collected from a single field in Athena, Oregon (USA), and isolate B3 was collected in Mt. Vernon, Washington State (USA). Molecular marker analysis using amplified fragment length polymorphisms (AFLPs) (Vos et al., 1995) indicated that all five isolates are genetically distinct (data not shown). The cultures were maintained on potato dextrose agar (PDA, Difco Inc., Detroit, MI) at room temperature.

Five pea lines were evaluated: PI 180693, 90-2079, Genie, Bolero and Dark Skin Perfection (DSP). PI 180693 and 90-2079 have been identified as resistant materials and used in previous investigations on the inheritance of resistance in pea to *A. euteiches* (Kraft and Boge, 1994; Wicker and Rouxel, 2001; Pilet-Nayal et al., 2002). The cultivars Genie (Novartis), DSP and Bolero are considered susceptible to *A. euteiches* (Kraft and Boge, 1994, 1996; Vandemark and Barker, 2003).

Inoculations and evaluation of disease severity

Zoospores of each isolate of *A. euteiches* were produced in a mineral salt solution (Llanos and Lockwood, 1960). Inoculum concentration was determined from a 5 ml aliquot that was shaken to induce zoospore encystment and counted with a hemacytometer.

Seedlings were inoculated with 10^5 zoospores per seedling. For all tests, pea seeds were surface-disinfested using an alcohol-hydrogen peroxide dip (Kraft and Erwin, 1967). Plants were grown with a 12-h photoperiod at 20–24 ± 2 °C. Seeds were planted in the greenhouse in flats containing coarse-grade perlite. For each isolate, an experiment consisted of a flat planted with one row of each pea line. All experiments were repeated. Five days after planting, the flats were thinned to a density of 7 plants per row. Seven days after planting, seedlings were inoculated by pipetting 5 ml of a 2×10^3 zoospore ml^{-1} suspension at the base of the plant. The flats were watered daily to maintain saturated conditions.

For each pea genotype × pathogen isolate combination, all seven plants were extracted from the flats 14 days after inoculations. The root systems were washed vigorously in tap water and the 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 the root system; 3 = brown discoloration on entire root system, but no symptoms on epicotyl or hypocotyl; 4 = brown discoloration on entire root system, and shriveled and brown epicotyl or hypocotyls; and 5 = dead plant. The entire root system of each plant was excised at the hypocotyls. DNA was subsequently extracted from the entire root system of each plant and analyzed with the real-time PCR assay.

DNA extraction

To extract DNA from individual plants, the entire root system of each plant was first rinsed in tap water and blotted dry on sterile paper towels. DNA was extracted from the entire root sample using the Fast-DNA kit (BIO 101, Inc., Carlsbad, CA) according to the manufacturer's recommendations. DNA was also isolated from roots of uninfected plants of both PI 180693 and Genie. DNA was also isolated as described previously (Vandemark et al., 2000) from pure cultures of each isolate of *A. euteiches* grown in PDB. DNA was quantified with a fluorometer (TD-700; Turner Designs, Inc., Sunnyvale, CA), and diluted to 20 ng μl^{-1} for use in quantitative PCR reactions.

Quantitation of A. euteiches *DNA in infected plants*

The nucleotide sequences of the forward primer, reverse primer and fluorochrome-labeled probe used to quantify *A. euteiches* are listed below. Forward primer 136F: 5′-GACTGCAATGTCG TCCAAGACTT-3′. Reverse primer 211R: 5′-ACAAAGCTGAGATGAAGAGATCGA-3′.Probe 161T: 5′-CAACCACCGAGCGAGCCGC-3′. The 5′ terminus of the probe (TaqMan; Applied Biosystems) was labeled with the fluorochrome 6-carboxyfluorescein (6FAM) and the 3′ terminus

was labeled with the quencher dye tetra-methylcarboxyrhodamine (TAMRA). The primer/probe set 136F-161T-211R amplified a 76-bp fragment. Primers and probe were synthesized commercially (Applied Biosystems). The specificity of this primer probe set towards *A. euteiches* and its inability to amplify DNA from uninoculated pea, uninoculated alfalfa, or other soilborne plant pathogens, including *Phytophthora medicaginis*, *Pythium* spp., *Verticillium albo-atrum*, *Fusarium* spp., and *Rhizoctonia solani*, have been previously demonstrated (Vandemark and Barker, 2003). DNA isolated from entire root systems of individual plants, both infected and healthy, 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. Reaction conditions and thermalcycling profiles were as described previously (Vandemark et al., 2002). Amplifications and detection of fluorescence were done using a GeneAmp 7000 Sequence Detection System (Applied Biosystems). Three replicate PCR reactions were performed on each DNA sample.

To quantify each isolate of *A. euteiches*, standard curves were constructed by including triplicate reactions containing pure DNA of the respective isolate that spanned six initial DNA quantities per reaction (0.01, 0.1, 1.0, 5, 25 and 100 ng). Standard curves plotted the $\log_{10}$ of DNA quantity on the *X*-axis and the PCR cycle at which the reaction crossed a threshold value of fluorescence (C_T) on the *Y*-axis. Reactions containing 100 ng of DNA isolated from uninfected plants of each pea line were also included to confirm that the primer/probe sets selectively amplified DNA from the pathogen genome and not from plant DNA.

Data analysis

Two independent experiments were conducted with treatment combinations consisting of isolate of *A. euteiches* and host genotype. Experiments were completely randomized and included seven replications. DSI and pathogen DNA content detected with real-time PCR were tested for normality by univariate analysis (Steel and Torrie, 1980). Mean comparisons were based on Fisher's protected LSD. The Spearman rank correlation between the DSI score and amount of pathogen DNA were calculated using the mean of three replicate quantitative PCR reactions for each plant sample. All procedures were performed using JMP Statistical Discovery Software (SAS Institute, Cary, NC).

Results

Sensitivity and specificity of the real-time PCR assay

The primer/probe set 136F-161T-211R detected DNA of all five isolates of *A. euteiches* in very linear assays within a range of 0.01–100 ng. The results were very similar for all isolates, with the correlation between the $\log_{10}$ of the initial DNA quantity and the C_T value ranging from –0.997 to –0.999. All PCR reactions containing 100 ng of DNA isolated from the roots of uninoculated plants had C_T values equal to 40, confirming that the primer/probe set 136F-161T-211R did not amplify DNA from healthy plants.

Relationship between DSI and quantity of A. euteiches *DNA in infected pea plants*

Results of analysis of variance (ANOVA) are presented for the quantity of *A. euteiches* DNA detected in pea plants using the real-time PCR assay and also for disease severity index (DSI) ratings of infected plants (Table 1). For both variables, significant differences were observed among host genotypes, pathogen isolates, host genotype × pathogen isolate interactions, pathogen isolate × experiment interactions, and host genotype × pathogen × experiment interactions. For the amount of pathogen DNA detected by the real-time PCR assay, significant differences were also observed for experiments and for host genotype × experiment interactions, while significant effects attributable to these sources were not observed for DSI.

A separate ANOVA was done that included only the four isolates collected in a single field from Oregon: C11, F3, D1 and E10. Similarly to the analysis with all five isolates, genotype × experiment and experiment effects were significant for pathogen DNA quantity, but not DSI (data not shown).

Results of comparisons of means between host genotypes for DSI are presented for all pathogen

Table 1. Analysis of variance for the quantity of *Aphanomyces euteiches* DNA detected in pea plants and disease severity index (DSI) ratings of infected plants

Source[1]	DNA (ng)			DSI	
	df	*F* value	Pr > *F*	*F* value	Pr > *F*
Host genotype (G)	4	122.66	< 0.0001	169.58	< 0.0001
Isolate (I)	4	45.29	< 0.0001	8.89	< 0.0001
Genotype × isolate	16	7.16	< 0.0001	6.15	< 0.0001
Experiment (E)	1	39.9	< 0.0001	1.93	0.1662
Genotype × experiment	4	3.91	0.0037	1.74	0.1409
Isolate × experiment	4	45.37	< 0.0001	13.55	< 0.0001
G × I × E	16	5.80	< 0.0001	2.21	0.0053

[1]Five pea genotypes were examined: PI 180693, 90-2079, Bolero, DSP and Genie. Two independent experiments were conducted. Five isolates of *A. euteiches*: C11, F3, D1, E10 and B3 were used in the study.

isolates (Table 2). For each pathogen isolate, PI 180693 had significantly lower DSI ratings than all other genotypes and 90-2079 had the second lowest mean DSI. For each pathogen isolate, DSP, Genie, and Bolero tended to have significantly higher mean DSI ratings than 90-2079. DSP and Bolero each were in the highest significant mean class for four of five pathogen isolates.

Table 2. Comparison of means[1] for disease severity index (DSI) ratings for different pea genotypes inoculated with isolates of *Aphanomyces euteiches*

Genotype	*A. euteiches* isolate				
	C11	F3	D1	E10	B3
DSP	4.43 a	4.02 a	4.07 a	4.54 a	3.21 a
Genie	3.57 b	4.07 a	3.29 b	4.07 a	4.14 b
Bolero	3.43 b	3.79 a	4.14 a	4.29 a	4.14 b
90-2079	2.57 c	2.71 b	3.29 b	3.07 b	2.14 c
PI 180693	1.51 d	1.92 c	2.5 c	1.86 c	1.71 c
LSD	0.54	0.52	0.44	0.30	0.50

[1]Data are presented in each column for a combined analysis of two replicate experiments. n = 14 plants of each genotype for each pathogen isolate. Means within a column that contain the same letter are not significantly different ($\alpha = 0.05$) based on Fisher's protected least significant difference (LSD). Each plant was scored using a DSI 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 the root system; 3 = brown discoloration on entire root system, but no symptoms on epicotyl or hypocotyl; 4 = brown discoloration on entire root system, and shriveled and brown epicotyl or hypocotyls; and 5 = dead plant.

Comparisons of means between host genotypes for the amount of pathogen DNA detected in plants are presented for all pathogen isolates (Table 3). For all pathogen isolates, the least amount of pathogen DNA was in 90-2079, and the highest amount was in Genie. Except for isolate F3, there was less pathogen DNA in 90-2079 than in PI 180693. Except for the cases of isolates D1 and E10, less pathogen DNA was detected in PI 180693 than in DSP or Genie. Differences between Bolero and DSP were generally not significant. The Spearman rank correlation (Table 2) between DSI and the amount of *A. euteiches* DNA detected in inoculated plants is presented (Table 3). The correlation was positive and significant for isolates C11, F3, and B3. However, this correlation was not significant for isolates D1 and E10.

Discussion

The relationship between disease severity and the amount of *A. euteiches* DNA detected in infected pea plants had been examined using with a precise and specific real-time PCR assay that measures pathogen DNA content in infected roots. These results should complement those of several previous investigations that have examined *A. euteiches* accumulation in pea over the course of disease development. These previous investigations used diverse quantification methods, including ELISA (Kraft and Boge, 1994), fatty acids analysis (Larsen et al., 2000), fungal specific enzymatic

Table 3. Comparison of means[a] between pea genotypes for the quantity (ng) of *Aphanomyces euteiches* DNA detected in plants inoculated with different pathogen isolates. The Spearman rank correlations (ρ) between DSI and pathogen DNA quantity are presented

Genotype	*A. euteiches* isolate				
	C11	F3	D1	E10	B3
DSP	2.01 a	1.63 a	0.98 bc	1.53 b	1.23 c
Genie	2.19 a	2.27 b	1.20 a	1.80 a	2.03 a
Bolero	1.39 b	1.54 a	1.08 ab	1.56 b	1.26 c
90-2079	0.89 c	1.09 c	0.68 d	0.57 c	0.66 d
PI 180693	1.40 b	1.22 c	0.87 c	1.56 b	1.58 b
LSD	0.31	0.28	0.15	0.18	0.33
ρ (Prob > $\lvert\rho\rvert$)	0.41 (0.0004)	0.42 (0.0003)	0.05 (0.6778)	−0.04 (0.8104)	0.26 (0.0302)

[a]Data are presented in each column for a combined analysis of two replicate experiments. n = 14 plants of each genotype for each pathogen isolate. Means within a column followed by the same letter are not significantly different (α = 0.05) based on Fisher's protected Least Significant Difference (LSD). 100 ng of DNA extracted from entire root system of infected plants was tested using the primer/probe set 136F-161T-211R. Each plant sample was tested with three replicate PCR reactions.

activity (Kjøller and Rosendahl, 1998), and real-time PCR (Vandemark and Barker, 2003). The results above, although very informative, are all based on experiments that each only used one isolate of *A. euteiches*. In this report, we describe results for an analysis of five pea genotypes with five isolates of *A. euteiches*.

Results of ANOVA for both DNA content and DSI (Table 1) indicate that host genotypes performed more consistently when evaluations were based on DSI ratings as opposed to DNA quantity. Significant differences were observed for experiments and for host genotype × experiment interactions for the amount of pathogen DNA detected by the real-time PCR assay, while these sources of variation were not significant for DSI. This indicates that the amount of DNA quantity in infected roots was more influenced by experimental conditions and genotype × experiment effects than DSI ratings. In previous experiments using real-time PCR to quantify *A. euteiches* DNA in pea and alfalfa (Vandemark et al., 2002; Vandemark and Barker, 2003), non-significant effects attributable to experiments and genotype × experiment interactions were observed, although significant effects were observed for alfalfa populations inoculated with *A. euteiches* WAPH-1 (Vandemark et al., 2002). Significant differences were observed for all other sources of variation, including pathogen isolate, for both DNA content and DSI (Table 1).

Much less pathogen DNA was detected in 90-2079 and Bolero than was previously detected in these genotypes with *A. euteiches* isolate SP7 (Vandemark and Barker, 2003). The mean amount of pathogen DNA detected in either population did not exceed 1.56 ng (Table 3). However, when *A. euteiches* isolate SP7 was used, the mean amounts of pathogen DNA detected in 90-2079 and Bolero were 4.86 and 11.24 ng, respectively (Vandemark and Barker, 2003). The highest DSI values for 90-2079 and Bolero were 3.29 and 4.29, respectively (Table 2), while the DSI values for 90-2079 and Bolero in response to isolate SP7 were 3.54 and 4.79 (Vandemark and Barker, 2003). These observations suggest that isolate SP7 causes more severe disease and more aggressively colonizes pea roots than the isolates included in the present study.

For three isolates, C11, F3 and B3, the correlation between pathogen DNA content and disease severity was positive and significant (Table 3). These results agree with previous observations using *A. euteiches* isolate SP7, the moderately resistant germplasm 90-2079, and the susceptible cultivar Bolero (Vandemark and Barker, 2003). The data presented in Tables 2 and 3 demonstrate that the susceptible genotypes Genie, DSP and Bolero tended to have significantly higher DSI and pathogen DNA content than the more resistant genotypes 90-2079 and PI 180693. These results also are similar to results seen for alfalfa and *A. euteiches*, where

screening many germplasm accessions and cultivars for reaction to several isolates of *A. euteiches* has consistently resulted in positive and significant correlations between pathogen DNA content and DSI (Vandemark et al., 2002; Vandemark and Barker, 2003).

However, for two of the pathogen isolates, D1 and E10, the correlation between DSI and pathogen DNA content was not significant (Table 3). The lack of significant correlation between DSI and pathogen DNA content for isolates D1 and E10 suggests that, in these cases, more pathogen was present in some of the plants, in particular PI 180693, which had low DSI ratings, than in other plants having higher DSI ratings. The lack of significance for isolates D1 and E10 suggests that the real-time PCR assay may have limited application as a selection tool for resistance in pea to *A. euteiches*. The utility of the assay as a selection tool would be dependent on the correlation between DSI and pathogen DNA content for the particular pathogen isolate. It may be necessary to investigate this relationship for a given isolate prior to dependence on the assay as a primary selection tool.

PI 180693 and 90-2079 had the lowest and second lowest DSI values respectively (Table 2), although this relationship is reversed in Table 3, as 90-2079 typically had significantly lower pathogen DNA content than PI 180693. Kjøller and Rosendahl (1998) observed that *A. euteiches* reduced mycelial formation and increased oospore production as disease progressed. The relatively healthy roots of PI 180693 may have afforded conditions conducive to mycelial growth rather than oospore formation. Actively growing mycelium probably has more nuclei per weight than oospores, which would result in higher pathogen DNA yields from infected tissue. Since the DSI ratings for 90-2079 were higher than for PI 180693, less pathogen DNA may have been present in 90-2079 because more severe root disease induced the pathogen to produce oospores rather than vegetative mycelia. Future studies examining the relationship between pathogen DNA content detected by real-time PCR and the relative abundance of mycelia and oospores in infected tissue would be informative. Cultivation of a pea genotype that expressed disease resistance but supported extensive production of oospores would have a detrimental effect towards long-term disease control in the field.

An alternative explanation for lower pathogen DNA content in 90-2079 is that this genotype expresses resistance in part by directly inhibiting pathogen colonization. Kraft and Boge (1994) detected significantly less *A. euteiches* in PI 180693 (resistant) than in DSP (susceptible) based on ELISA, and suggested that reduced pathogen growth was a characteristic of resistance. The many examples of positive and significant correlations between DSI and *A. euteiches* DNA content in infected pea and alfalfa presented in this study and previously (Vandemark et al., 2002; Vandemark and Barker, 2003) also provide evidence that an inhibition of pathogen growth is often associated with the expression of resistance in both alfalfa and pea to *A. euteiches*.

PI 180693 consistently had the lowest DSI of all host genotypes for all pathogen isolates, but also consistently had higher quantities of pathogen DNA than 90-2079. These results suggest that disease development in PI 180693 may be inhibited in part by a mechanism that confers tolerance to the effects of colonization by *A. euteiches*. These results also suggest an alternative application of the real-time PCR assay in identifying avirulent or mildly virulent isolates of *A. euteiches*. These isolates would be characterized by high levels of DNA detected in host tissue in the absence of disease symptoms.

The real-time PCR assay should have considerable applications in the study of microbial interactions in peas. It may serve as a complementary tool to fatty acid analysis for studying the interaction in pea between *A. euteiches* and *Glomus. mosseae* (Larsen and Bodker, 2001). It could also be used in a multiplex-PCR reaction to examine interactions between *A. euteiches* and other pathogens responsible for seedling diseases in pea, including *Fusarium solani*, *Rhizoctonia solani* and *Pythium ultimum*.

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