

Evaluation of Downy Mildew Resistance in Spinach (*Spinacia oleracea*)

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Abstract

Spinach (*Spinacia oleracea*) is an economically important leafy vegetable grown in the United States and world-wide. The downy mildew pathogen, *Peronospora effusa* (*Pfs*), is a major biotic constraint impacting spinach production and quality. The use of resistant cultivars is an economical management option especially in organic production systems. As new races of the pathogen continue to appear, there is a need to continue to select for resistance to the emerging races. The objectives of this study were to evaluate a set of spinach hybrids and F2 breeding populations for resistance to *Pfs* race 5 to develop a better understanding of the genetics of downy mildew resistance. Also, we screened 39 commercial spinach cultivars for resistance to a recently identified race, *Pfs* race 19. The genetics of resistance to *Pfs* 5 was determined by greenhouse inoculations of F1 progeny (individual crosses between near isogenic lines, NIL3 or NIL1 and susceptible genotype, Viroflay) and F2 population progeny (cross between Califlay and susceptible Viroflay). Two hybrids were examined for resistance to *Pfs* 5. The results indicated that resistance conferred at the *RPF1* or the *RPF3* loci in a heterozygous (Rr) condition to *Pfs* 5 was completely dominant. Also, Chi-square analysis of the segregation pattern in the F2 population showed that resistance to *Pfs* race 5 was conferred by a single dominant gene. A total of 22 out of 39 spinach commercial cultivars were resistant to the newly reported *Pfs* race 19 and could be used in breeding programs to develop new cultivars with resistance to *Pfs* 19.

Introduction

Downy mildew in spinach (*Spinacia oleracea* L.), caused by the obligate oomycete *Peronospora effusa* (*Peronospora farinosa* f. sp. *Spinaciae*, *Pfs*), is a major disease threatening spinach production globally. The management of downy mildew on spinach involves the use of resistant cultivars, fungicides, crop rotation, or a combination of approaches to manage the disease. Since the first report of the pathogen on spinach (Greville 1824), 18 additional races of the downy mildew pathogen have been reported (Correll and Smilde 2021; Correll et al. 1994, 2011). The continual emergence of new races can be attributed to a number of factors including the strong selection pressure on the pathogen with the release of resistant cultivars. (Correll and Smilde 2021; Dhillon et al. 2020a; Feng et al. 2018a; Kandel et al. 2019; Skiadas et al. 2022). Currently, a total of 19 *Pfs* races have been identified and described with race 5 reported in 2003 (Feng et al. 2018a; Irish et al. 2003) and race 18 and 19 recently denominated by the International Working Group on spinach downy mildew (Correll and Smilde 2021; Feng et al. 2021). It is expected that the rapid emergence of novel races of this pathogen will continue to impact spinach production (Correll et al. 2011).

Among the methods of plant disease management, the use of genetic resistance is a sustainable, economical and environmentally friendly method typically adopted in many organic (spinach) production systems. This involves the use of resistant cultivars for the control of diseases (Branham et al. 2021; Choudhury and McRoberts 2020; Correll et al. 2011; Saha et al. 2020). Developing and improving resistance to the downy mildew pathogen is one of the primary goals in most spinach breeding programs (Bhattarai and Shi 2021; Correll et al. 2011; Morelock and Correll 2008). Several efforts have focused on

dissecting the genetic basis of downy mildew resistance and developing genetic resources that could be incorporated in spinach breeding programs (Bhattarai et al. 2020a, b; 2021; Brandenberger et al. 1992; Eenink 1976; Feng et al. 2018a; Irish et al. 2008). Earlier studies had suggested resistance to two *Pfs* races (1 & 3) are conferred by two closely linked genes (Eenink 1976). To study the genetics of resistance, Irish et al. (2008) developed open-pollinated near-isogenic lines each having a single resistance locus that could be used for genetic studies. Particularly, a near isogenic line (NIL1) was developed from a cross between a homozygous susceptible Viroflay as recurrent parent and resistant parent (Lion). Lion has resistance to many *Pfs* races, including race 6 which was one of the prevalent *Pfs* races at the time. Briefly, female Lion plants and male Viroflay plants were crossed in a controlled (greenhouse) system. Then, seeds from individual plants were harvested as bulked F1 populations. A total of 218 F1 plants were initially screened to *Pfs* race 6 for disease reaction. Identified resistant F1 progenies were then backcrossed to Viroflay female plants and the process of inoculation and resistance identification was pursued through four backcrossed generations. Six resistant plants backcrossed four times were selected and selfed. Further, 5 of the 22 resistant plants derived from the selfed population were again selfed and progenies from 2 selfed lines were screened for resistance to *Pfs* 6 and for homozygosity at a locus (designated *RPF1*) based on genetic analysis of resistance in the progeny. In the study, they established that NIL1 plants are homozygous resistant at the *RPF1* locus and that crosses between NIL1 and Viroflay, gives hybrids that are heterozygous resistant at the *RPF1* locus. Also, they showed that the *RPF1* locus segregates as a single dominant locus. In a recent study, Feng et al. (2018) investigated *RPF1* and two other loci (*RPF2* and *RPF3*). Like Irish et al. (2008), the study adopted Lion as the resistance source of *RPF1*. For the *RPF2* and *RPF3* loci, the study used Lazio and Califlay as resistance sources, respectively. For all the three *RPF* loci, the universally susceptible Viroflay was used as recurrent parent in three backcrosses for F1 progenies of each of the crosses involving Viroflay and the three resistance loci (*RPF1*, 2 & 3) sources (Lazio, Lazio and Califlay). Results from their study showed that each *RPF* locus segregates as a single dominant locus for all *Pfs* races tested. Efforts have been geared towards fine-mapping the *RPF1* (Bhattarai et al. 2020b; 2021; 2022; She et al. 2018; Yang 2013), with other loci in the pipeline for fine-mapping.

Resistance characterization to the various races of the pathogen (*Pfs*) has led to the identification of resistance sources (Bhattarai et al. 2020a; Brandenberger et al. 1992; Feng et al. 2018a; Irish et al. 2003; 2007). The adoption of a standard set of differential genotypes possessing putative resistance loci (*RPF*) robust for discriminating *Pfs* races based on the phenotypic disease response on whole plant inoculation assays, are standardized for identifying resistance to the pathogen (Feng et al. 2018a ; Irish et al. 2003). Recently, Bhattarai et al. (2020a) reported a detached leaf assay method which could be used alternatively for characterizing resistance to *Pfs* in spinach. At least, six major resistance (*RPF*) loci were hypothesized and reported to control resistance to the described races of the pathogen (Correll et al. 2011). The *RPF1* is reported to confer resistance to at least 12 of the 19 documented races. Similarly, *RPF2* confers resistance to at least 11 races, while *RPF3* confers resistance to more than nine races of the downy mildew pathogen. Among the resistant spinach genotypes, a number of spinach genotypes or cultivars with resistance alleles at some identified loci (*RPF1*, 2, 3 and 6), have been identified to possess

resistance to *Pfs* race 5 (Irish et al. 2003; 2008). The latest denominated race, *Pfs* 19 has been reported to infect most differentials (NIL1,2,4,5,6, Pigeon, Meerkat and Hydrus) but not those with putative resistance close to the *RPF3* locus like NIL3 (Bhattarai et al, 2020a; <https://ucanr.edu/blogs/blogcore/postdetail.cfm?postnum=46392>). The development of spinach genotypes potentially containing putative *RPF* loci (1–6) would provide a durable form of resistance to the reported races of the downy mildew pathogen.

Despite the progress on the genetic basis of downy mildew resistance, there is still a need to further understand the genetics of resistance at the reported *RPF* loci. Observations suggested that the resistance response in some spinach genotypes containing the putative *RPF* loci was not clear in that heterozygous (Rr) resistance was not completely dominant and allowed some infection to occur (Correll et al. 2011; Correll, personal communication). Based on discussions with spinach breeders from private industry, we sought to understand if resistance conferred at the *RPF3* locus in the heterozygous condition (Rr) showed complete dominance. An incomplete resistance to *Pfs* is defined by plants often showing symptoms and sporulation on cotyledons but not on true leaves (Correll et al. 2011; Irish et al. 2003). The evaluation of spinach genotypes at a locus with known allelic combinations can provide more information on incomplete resistance to *Pfs* races. The objectives of this study were to evaluate a set of hybrids and F2 breeding populations for resistance to *Pfs* race 5, and to characterize resistance to the newly reported *Pfs* race 19 in commercial cultivars with resistance alleles at most *RPF* loci.

Materials and methods

1. Evaluation of an F1 (NIL3 x Viroflay) and F2 (Viroflay x Califlay) population progenies for resistance to *Pfs* race 5

Plant materials, planting, inoculation and rating

In the first experiment of the study, we evaluated two spinach hybrids (NIL1 x Viroflay and NIL3 x Viroflay) for incomplete resistance response to *Pfs* race 5. The hybrids were each developed from a cross between Viroflay (identified to be universally susceptible to all reported *Pfs* races), and individual open-pollinated near-isogenic lines, NIL1 and NIL3. NIL1 is resistant to *Pfs* race 5 and reported to have resistance allele at the *RPF1* locus. Similarly, NIL3 is resistant to *Pfs* race 5 and reported to possess resistance allele at the *RPF3* locus. The hybrids (NIL1 x Viroflay and NIL3 x Viroflay), together with checks, Califly and Lion, were considered in this evaluation (Table 1). Califlay has resistance allele at the *RPF3* locus while Lion has resistance allele at both *RPF1* and *RPF3* loci (Feng et al. 2018a). Plants from the genotypes were established by direct seeding in 3-inch pottymix-filled pots in three replicates (pots) per cultivar. Each replicate had five plants, making a total of 15 plants per genotype in a trial. The evaluation was conducted in six independent trials. The plants were grown in the greenhouse (of the Rosen Center at the University of Arkansas, Fayetteville, Arkansas) following standard procedure for two weeks and inoculated following the routine inoculation method (Feng et al. 2014) (Fig. 1).

Table 1

Disease response of a set of spinach genotypes to downy mildew *Pfs* race 5 under greenhouse conditions in six independent tests

Cotyledon infection ^a						
Genotype	<i>RPF1</i> Alleles	<i>RPF3</i> Alleles	Expected	Observed	Disease incidence (%)	Disease severity (%) ^b
Viroflay	rr	rr	+	+	100.0	87.2
Califlay	rr	RR	-	-	0.0	0.0
F1 (NIL3 x Viroflay) ^c	rr	Rr	?	-	0.0	0.0
F1 (NIL1 x Viroflay) ^c	Rr	rr	-	-	0.0	0.0
NIL3	rr	RR	-	-	0.0	0.0
NIL1	RR	rr	-	-	0.0	0.0
Lion	Rr	Rr	-	-	0.0	0.0
^a Each test consisted of 3 replicates with 15 plants per genotype. Cotyledons: '+' indicates > 85% of the seedlings showed evidence of infection and sporulation after 7 days, '-' indicates < 15% of the seedlings showed evidence of infection and sporulation after 7 days. ? = means reaction undetermined						
^b Disease severity was calculated using the mean disease response (severity) of all replicates in all tests for each genotype based on true leaf infection. True leaves were rated on a scale of 0 to 4, 0 (no symptom and 0% disease severity), 1 (1–25% chlorosis and necrosis), 2 (26–50% chlorosis and necrosis), 3 (51–75% chlorosis and necrosis), and 4 (76–100% chlorosis and necrosis) respectively.						
^c The F1 is used for investing for incomplete resistance.						
RR = homozygous resistance; Rr = heterozygous resistance; rr = homozygous susceptible						

Briefly, *Pfs* sporangia were retrieved from sporulating spinach leaves and filtered through four layers of cheesecloth. Each pot of plants was inoculated with 20 ml (10⁵ sporangia/ml) of inoculum using a Badger basic spray gun (model 250). The inoculated plants were immediately transferred to the dew chamber (100% relative humidity) for incubation at 18°C for 24 h. The plants were then moved to the growth chamber at 19°C with a 12 h light/12 h dark regime for 5 days and transferred back to the dew chamber for 24 h to induce sporulation. Qualitative evaluation was based on the presence or absence of sporulation on cotyledons and true leaves; as resistant (-) or as susceptible (+), respectively. Quantitative evaluation of true leaves was conducted as previously described (Irish et al. 2003). Briefly true leaves were evaluated on a standard scale of 0 to 4, with 0 = no sporulation; 1 = up to 25% leaf area covered with sporulation; 2 = 26 to 50% leaf area covered with sporulation; 3 = 51 to 75% leaf area covered with

sporulation; and 4 = 76 to 100% leaf area covered with sporulation. The disease incidence and severity on each cultivar was calculated using the standard method as thus:

Downy mildew disease incidence (DI) on the genotypes was calculated based on the number of cotyledons infected: + indicates > 85% infected, - indicates < 15% infected, and $\pm$ indicates an intermediate reaction. Disease severity (DS) on the cultivars was calculated using the mid-point of the range of each disease category (0–4) with the formula: $DS = [(A \times 0) + (B \times 12.5) + (C \times 37.5) + (D \times 62.5) + (E \times 87.5)] / (A + B + C + D + E)$, where A, B, C, D, and E represents (0–4). The downy mildew response data (severity) was used for assessing the degree of resistance and susceptibility to estimate or capture resistance level in the hybrid.

Evaluation of F2 population progenies

For the second experiment of the study, two F2 population progenies from a cross of Viroflay x Califlay consisting of 142 and 63 individuals, and parents (Viroflay and Califlay) were evaluated in the greenhouse of the Rosen Center at the University of Arkansas, Fayetteville, Arkansas. Califlay is identified to be resistant to *Pfs* race 5 and reported to possess resistance allele at the *RPF3* locus. The F2 breeding populations were developed in USDA, California by Dr. Beiquan Mou. In this study, plants were grown following standard procedure by direct seeding in rows inside 25 cm x 50 cm plastic trays filled with the sunshine potting soil (Sun Gro Horticulture, Canada). Seeds were planted in 10 rows per tray and 15–20 seeds per row. Two trays were used for each population with each tray receiving at least 120 seeds of the F2 populations. The parents were grown by direct seeding as well in a tray. The inoculation protocol for downy mildew followed the standard procedure and plants were scored qualitatively based on the cotyledon infection assay (Feng et al. 2014; Irish et al. 2003).

2. Characterization of resistance to *Pfs* race 19 in commercial cultivars

Plant materials, planting, inoculation and rating

In this experiment, a selected set of 39 contemporary commercial cultivars with sources of resistance to the previously described races was evaluated under greenhouse/growth chamber conditions at the Rosen Center of the University of Arkansas, Fayetteville. The commercial cultivars were part of the panel previously evaluated in the field to the prevalent *Pfs* races in California and Arizona (Clark et al. 2020; Dhillon et al. 2019; 2020b.). Based on the standard pathogenicity assay, it is hypothesized that the *RPF3* locus confers resistance to *Pfs* race 5 (Feng et al. 2018b). Thus, at least 3 different cultivar/genotypes (Califlay, NIL3, and Whale) known to contain resistance allele(s) at the *RPF3* locus were used as negative control, and one cultivar (Viroflay) known to be universally susceptible to all known *Pfs* races, was considered as positive control.

To characterize downy mildew resistance in the commercial cultivars, the experiment was carried out in two independent trials. Plants were grown in rows inside trays for each test with each tray containing positive (Viroflay) and negative (Califlay) controls. At least, two-week-old seedlings were used for

inoculations in this study and plants were inoculated following standard procedure (Feng et al. 2014; Irish et al 2003). Disease severity (DS) on the cultivars was calculated using the mid-point of the range of each disease category (0–4) with the formula: $DS = [(A \times 0) + (B \times 12.5) + (C \times 37.5) + (D \times 62.5) + (E \times 87.5)] / (A + B + C + D + E)$, where A, B, C, D, and E represents (0–4) as described previously.

Data analysis

The resulting downy mildew response data were subjected to descriptive statistical analysis and analysis of variance. The analysis was done in EXCEL (Microsoft) and JMP Pro 15 (SAS Institute, Cary NC). The Tukey's test (Honestly Significant Difference) was used for the mean separation at $P = 0.05$

Results

1. Evaluation of a hybrid (NIL3 x Viroflay) and F2 (Viroflay x Califlay) population progenies for resistance to *Pfs* race 5

In the first experiment, attention concentrated on evaluating a hybrid (NIL3 x Viroflay) for downy mildew response upon inoculation with *Pfs* race 5 under greenhouse conditions. Based on the data generated from this study, Viroflay was susceptible to *Pfs* race 5 as expected, while all other genotypes including the hybrid (NIL3 x Viroflay) showed complete resistance (Table 1). The result was consistent across the entire six independent trials conducted in this study. This indicates that the resistance imparted against *Pfs* race 5 is completely dominant when the *RPF3* resistance alleles are present in a heterozygous condition in the F1 hybrid, NIL3 x Viroflay.

In the second experiment, we evaluated two F2 population progenies from a cross of Viroflay (susceptible) x Califlay (resistant) segregating for resistance to *Pfs* race 5 under greenhouse conditions based on qualitative cotyledon infection assay. In the first progeny population with 142 individuals, a total of 102 individual plants were found resistant while 42 were susceptible to *Pfs* race 5 of the downy mildew pathogen (Table 2). Similarly, a total of 49 plants were resistant while 14 were susceptible in another Viroflay x Califlay F2 progeny (Table 2). The segregation pattern analysis of the downy mildew response in the Viroflay x Califlay F2 populations fit a 3:1 ratio of resistance to susceptibility, indicating that the response observed fit the expected model of single gene resistance based on chi-square analysis (P value = 0.38 and 0.62, respectively in each F2 progenies, and $P = 0.65$ combined) (Table 2).

Table 2

Disease response of Viroflay x Califlay F2 populations to *Pfs* race 5 under greenhouse conditions in two independent tests

		Disease reaction ^c				
Parentage ^a	Progeny panel ^b	Resistant	Susceptible	Segregation	χ^2	P
Viroflay x Califlay	I	102	40	3:1	0.76	0.38
	II	49	14	3:1	0.25	0.62
	Combined	151	54	3:1	0.19	0.65
^a The genetic background of the F2 population considered in this study						
^b Each F2 biparental population progeny was considered an independent test (I or II)						
^c Observed disease response.						
Chi-square values (χ^2) were not significantly different from a 3:1 segregation ratio if < 3.84 at the $P = 0.05$ level.						

2. Characterization of resistance to *Pfs* race 19 in commercial cultivars

In third experiment of the study, the disease response to downy mildew *Pfs* race 19 among 39 spinach cultivars was evaluated both qualitatively and quantitatively. Data from the qualitative method based on cotyledon infection assay revealed 22 of the tested cultivars to be resistant while 17 were susceptible in two independent trials (Table 3). For quantitative evaluation, the downy mildew disease response observed on the cultivars was similar in the two trials (Table 3) and analysis of variance test indicated significant differences in the cultivars at $P < 0.0001$. As expected, Viroflay showed consistent and complete susceptibility to the race 19 of the pathogen, while Califlay was completely resistant. Other than Viroflay, a cultivar (Red Kitten) was notably susceptible to the race of the pathogen in the two trials. The cultivars were categorized into two classes (resistant and susceptible) when the means were separated based on Tukey's test ($\alpha = 0.05$) (Table 3). Essentially, the spinach cultivars in this study showed either complete resistance or susceptibility to *Pfs* race 19.

Table 3. Disease reactions of commercial spinach cultivars inoculated with *Pfs* race 19 in two independent greenhouse inoculation tests.

Cultivar	Source	Cotyledon ^a		True leaves ^b		Mean
		Test 1	Test 2	Test 1	Test 2	
						Severity ^c
Califlay	U of A	-	-	0.0	0.0	0.0a
C7-613	Sakata	-	-	0.0	0.0	0.0a
Cabazon	RZ	-	-	0.0	0.0	0.0a
Crater	BASF	-	-	0.0	0.0	0.0a
Escalade	ENZA	-	-	0.0	0.0	0.0a
Lakeside	Sakata	-	-	0.0	0.0	0.0a
Minkar	BASF	-	-	0.0	0.0	0.0a
Riverside	Sakata	-	-	0.0	0.0	0.0a
Seaside	Sakata	-	-	0.0	0.0	0.0a
Spoonbill	RZ	-	-	0.0	0.0	0.0a
Sunangel	RZ	-	-	0.0	0.0	0.0a
SV1864VC	Bayer	-	-	0.0	0.0	0.0a
Trailboss	ENZA	-	-	0.0	0.0	0.0a
Yukon	ENZA	-	-	0.0	0.0	0.0a
Whale	RZ	-	-	0.0	0.0	0.0a
Crosstrek	ENZA	-	-	0.0	0.0	0.0a
Dracus	BASF	-	-	0.0	0.0	0.0a
Nembus	BASF	-	-	0.0	0.0	0.0a
Pawnee	PV	-	-	0.0	0.0	0.0a
Regor	BASF	-	-	0.0	0.0	0.0a
Tabit	BASF	-	-	0.0	0.0	0.0a
Tacoma	ENZA	-	-	0.0	0.0	0.0a
Alcor	BASF	+	+	68.0	81.6	74.8b
Midway	Bayer	+	+	87.5	87.5	87.5b
Patton	Bejo	+	+	87.5	87.5	87.5b
Platypus	RZ	+	+	81.3	87.5	84.4b

PV1506	PV	+	+	87.5	87.5	87.5b
PV1512/Bandera	PV	+	+	87.5	87.5	87.5b
PV1513/Dallas	PV	+	+	87.5	70.8	79.2b
PV1514/Laredo	PV	+	+	87.5	67.2	77.4b
Red Kitten	RZ	+	+	87.5	87.5	87.5b
SV2146VB	Bayer	+	+	87.5	87.5	87.5b
SV2157VB	Bayer	+	+	87.5	87.5	87.5b
SV3580VC	Bayer	+	+	87.5	87.5	87.5b
Viroflay	U of A	+	+	87.5	87.5	87.5b
Corvus	BASF	+	+	87.5	87.5	87.5b
El Prado	Syngenta	+	+	87.5	87.5	87.5b
Magnetic	Bayer	+	+	87.5	87.5	87.5b
Nevada	PV	+	+	87.5	87.5	87.5b
HSD _(0.05)	...	...	...	...	...	4.2

^aCotyledons: '+' indicates >85% of the seedlings showed evidence of infection and sporulation after 7 days, '-' indicates <15% of the seedlings showed evidence of infection and sporulation after 7 days and '+/-' indicates an intermediate reaction between 15% and 85%. True leaves were rated on a scale of 0 to 4, 0 (no symptom and 0% disease severity), 1 (1-25 % chlorosis and necrosis), 2 (26-50 % chlorosis and necrosis), 3 (51-75 % chlorosis and necrosis), and 4 (76-100 % chlorosis and necrosis) respectively. Numbers in parentheses indicate percentage of cotyledons that were susceptible.

^bDisease severity (DS) on true leaves (with at least 5 plants per genotype per test) was calculated using the mid-point of the range of each disease category (0-4) with the formula: $DS = [(A*0) + (B*12.5) + (C*37.5) + (D*62.5) + (E*87.5)] / (A+B+C+D+E)$, where A, B, C, D, and E represents each rating value (0-4) as described above.

^cCombined means of two tests. Means followed by the same letter in the column are not significantly different following the Tukey's test at $\alpha=0.05$.

Discussion

Resistance to pathogens is generally influenced by several factors including the interaction between the host plant and the pathogen. Based on field reports citing intermediate or incomplete resistance in some

spinach hybrids with putative resistance alleles in heterozygous combination, we focused on investigating the nature or level of resistance conferred on a spinach hybrid upon artificial inoculation using a known *Pfs* race (race 5). We presented a hybrid (NIL3 x Viroflay) with resistance conferred via the parent (NIL3) by a single gene with hypothesis that the 'single' resistance gene from the parent (NIL3) may show incomplete penetrance when present in heterozygous combination at the *RPF3* locus, and can lead to incomplete dominant resistance. In this study, the inoculation of the hybrid (NIL3 x Viroflay) with *Pfs* race 5 resulted in complete resistance (disease severity of 0%) indicating a complete dominant gene effect when *Pfs* race 5 was used for inoculation. The genetic architecture of resistance alleles at respective *RPF* loci is not fully understood and this can impact the understanding of the type and level of resistance conferred to a given race of the downy mildew pathogen. Generally, downy mildew resistance is deemed complete when resistance alleles are in heterozygous combination in hybrid or when resistance alleles are in homozygous combination in resistant parent (Correll et al. 2011; Irish et al. 2008). Hence, considerable attention should be placed on understanding the mode of resistance conferment in the genetic background used for developing a hybrid to ensure durable resistance to a given *Pfs* race. Partial resistance is widely observed in different crops including vegetables. For example, resistance to tomato spotted wilt virus in tomato (Roselló et al. 1998) and resistance to *Bremia lactucae* in lettuce (Crute and Norwood 1978). While results from this current study can be considered reliable, the use of more races in evaluating this genotype (NIL3 x Viroflay) and other hybrids can provide more information about the mode of resistance to *Pfs* races in spinach. As advances are made towards cloning the downy mildew resistance gene(s) in spinach, the potential cloning of the underlying resistance gene(s) will aid the better understanding of the type of interaction (compatible/incompatible) existing between *Pfs* races and spinach genotypes, which drives resistance and susceptibility. Further, functional analysis of cloned resistance genes will shed light on the molecular mechanism underlying resistance and susceptibility existing in spinach-*P. effusa* pathosystem. Similarly, it would potentially reveal the events driving the constant evolution of *Pfs* races (Bhattarai and Shi 2022; Bhattarai et al. 2020b; Correll et al. 2011; Crute 1992).

In this study, the response of the F2 population progenies fits the expected segregation ratio for a trait controlled by single dominant R gene. In spinach, resistance was previously characterized to be controlled by a single dominant gene (Smith 1950; Smith et al. 1961) and then later found to be conferred by closely linked genes (Eenink 1974; 1976). To resolve the complexity over downy mildew resistance pattern in spinach, Irish et al. (2008) identified a resistant locus (*RPF1*) in a developed near isogenic line previously phenotyped for downy mildew resistance. Their result indicated that the resistance identified in the genetic background of the near isogenic line segregates as a single dominant locus. However, it is hypothesized that the genetic architecture controlling downy mildew resistance in spinach is likely controlled by one or very few gene(s) and/or with multiple alleles (Correll et al. 2011). In the lettuce-*Bremia lactucae* pathosystems, downy mildew resistance was initially reported to be conferred by tightly linked genes (Hulbert and Michelmore 1985). Recently, there have been the identification of multiple quantitative trait loci conditioning resistance in addition to the previously identified major genes (Simko et al. 2015).

The emergence of a novel race/strain of the downy mildew pathogen can pose a considerable threat to spinach production. The various agronomic practices adopted in spinach growing regions allows for the constant emergence of new races that continues to threaten spinach production. For instance, the expanding spinach market has caused an increase in the production and the acreage cultivated which makes downy mildew disease occurrence possible in spinach fields all year round. Also, an oospore-infested spinach seed can potentially introduce a new race into an otherwise non-infested growing region (Correll et al. 2011). The biological system of *P. effusa* is another major concern as sexual recombination between *Pfs* races/strains can result to the emergence of new races. Importantly, this mode of reproduction has been implicated in the marked rise in the number of new races or deviating strains of the downy mildew pathogen identified in the past few decades (Correll et al. 2011; Dhillon et al. 2020; Skiadas et al. 2022). Thus, screening and breeding for improved resistance remains a priority in spinach programs to keep pace with the devastating pathogen (Bhattraai and Shi 2022; Bhattraai et al. 2020c). In this study, the response of the individual cultivar to the new race *Pfs* race 19 supported the existing theory on the genetics of resistance (single gene or few genes) to downy mildew pathogen in spinach.

Most spinach genotypes are usually susceptible to a novel race across all growing regions. Also, some genotypes can have a quantitative form of resistance to the novel race of the downy mildew pathogen – ranging from partial or incomplete to complete resistance depending on the genetic background of the genotype (Correll et al. 2011). Robust resistance characterization can aid the incorporation of resistance into diverse spinach genetic background such as open-pollinated cultivars or hybrid. Like response to the previous races of the pathogen, the response of the individual spinach cultivars in this study revealed that resistance to the new race is likely controlled by single gene (or alleles) and that very few genes or multiple alleles are likely involved in resistance conditioning to each race of the downy mildew pathogen, depending on the genetic background or source of resistance. As quantitative resistance exploration becomes more prominent in spinach, increased attention on intensive field evaluation for resistance characterization to the downy mildew pathogen would lead to the identification of a more robust and durable resistance sources (Bhattarai et al. 2020c; Clark et al. 2020; Correll et al. 2011; Dhillon et al. 2019; 2020; Irish et al. 2003). This would establish and provide a platform whereby multiple genes controlling resistance to all known races of the pathogen can be potentially incorporated into a genetic background.

Conclusion

The evaluation of downy mildew resistance in spinach was conducted by (1) screening a set of hybrids and biparental F2 population progenies for resistance to race 5 of the downy mildew pathogen, and (2) screening commercial cultivars for resistance to the new race, *Pfs* 19 using whole-plant pathogenicity assay. Disease incidence and severity were used as phenotypic traits to assess resistance. A completely dominant RPF3 allele was validated in Califlay for downy mildew resistance. Results from this study strengthened our understanding on the genetics of downy mildew resistance in spinach and revealed some cultivars that can be used to introgress locus/genes controlling resistance to the novel race (race 19) of the downy mildew pathogen in spinach.

Declarations

Author Contributions: A.S. and J.C. are the Project Investigators who conceived and lead the project. D.O., G.B., and C.F. collected the original phenotypic data. D.O. drafted the manuscript. G.B., J.C. and A.S. critically revised the manuscript. All authors have read and agreed to the final version of the manuscript.

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Figures

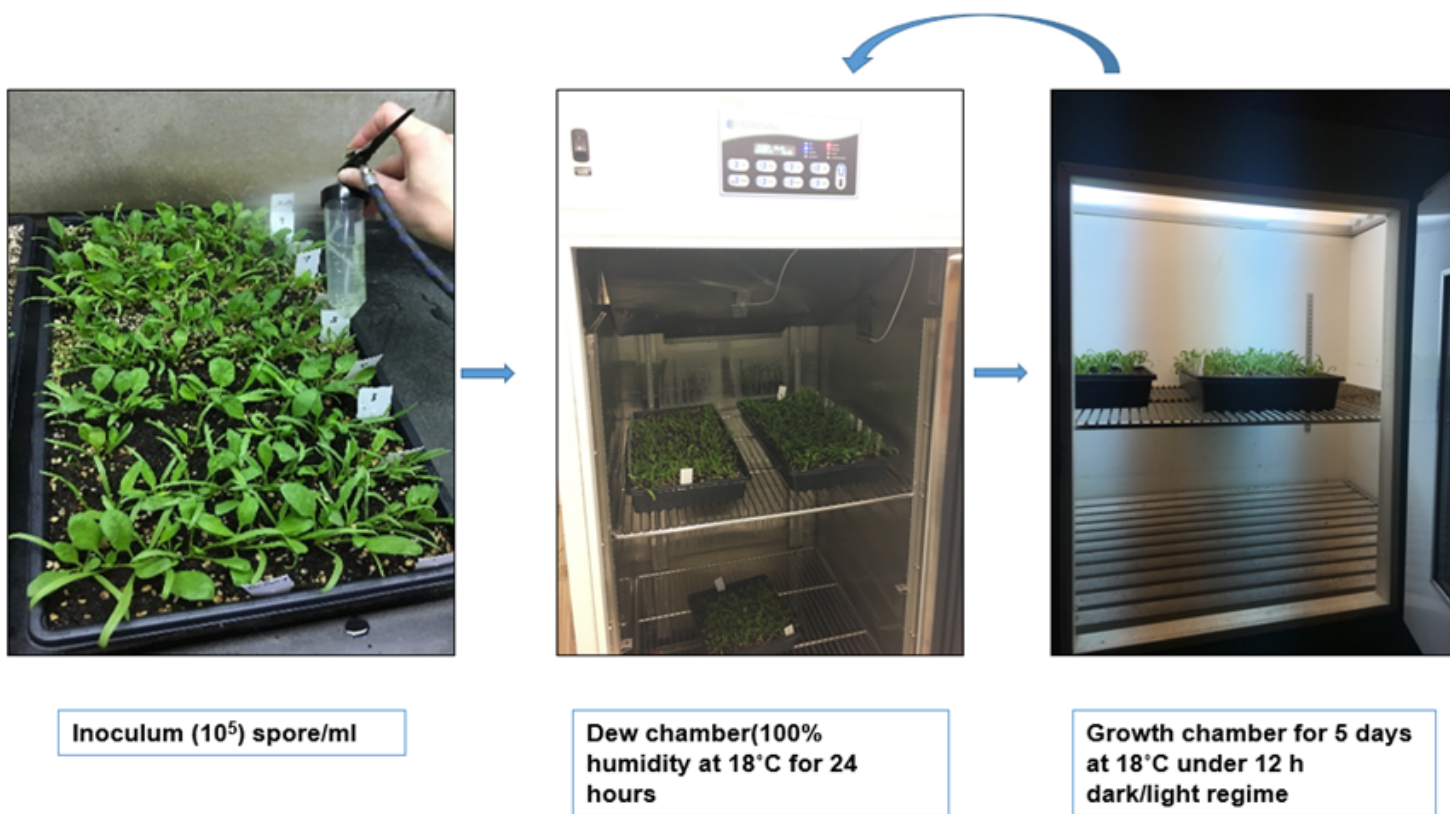


Figure 1

Downy mildew inoculation workflow in dew and growth chambers