Characterization of Partial Resistance to Black Spot Disease of *Rosa* sp.

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Abstract. Black spot disease, caused by the fungus *Diplocarpon rosae* Wolf, is one of the most serious diseases of garden roses. Both complete (vertical) resistance conditioned by dominant *Rdr* genes and partial (horizontal) resistance conditioned by multiple genes have been described. The use of resistant rose cultivars would reduce the demand of agrochemical applications. The characterization of 16 genotypes for resistance to black spot using two laboratory assays, the detached leaf assay (DLA) and the whole plant inoculation (WPI) approach, indicated that these techniques were well correlated. Thus, either method could be used to assess the resistance of the plants to black spot. Fifteen diploid hybrid populations from 10 parents segregating for partial (horizontal) resistance to black spot derived from *Rosa wichuraiana* ‘Basye’s Thornless’ (RW) were assessed for black spot resistance by quantifying the percentage of the leaf area with symptoms (LAS) and lesion length (LL) measured by the diameter of the largest lesion per leaf in DLAs. The narrow-sense heritability of partial resistance to black spot as measured by LAS and LL data of DLA was estimated to be from 0.28 to 0.43 when calculated with a genetic variance analysis and from 0.74 to 0.86 when generated from offspring–midparent regression. This suggests that the development of rose cultivars with high levels of stable partial resistance to black spot is a feasible approach for the rose industry.

Roses, which are distributed throughout the temperate regions of the Northern Hemisphere (Krüssmann, 1981), have been important ornamental plants for more than 5000 years. There are thousands of cultivars for the garden, floriculture, medicinal, fragrance, and culinary industries (Gudin, 2000; Marriott, 2003; Shepherd, 1954). The rose industry contributes a value of about \$400 million from garden roses, which is the major crop in the \$2.81 billion

wholesale U.S. shrub market (American Hort, 2014). Compared with the rose market in the United States 35 years ago, the production of garden roses has decreased (Byrne et al., 2010) from 40 million roses down to 12 million field-grown and 15–18 million pot-grown rose bushes in 2012 (Hutton, 2012). This is thought to be because many rose cultivars have low tolerance to disease and abiotic stress (Waliczek et al., 2015). In response to a consumer demand for low-maintenance roses, many rose breeding programs are working toward developing cultivars resistant to the common rose diseases (Byrne, 2015; Debener and Byrne, 2014).

Black spot, the most important disease affecting garden roses globally, is caused by the fungus *D. rosae* Wolf (*Marssonina rosae* anamorph) (Nauta and Spooner, 2000). The typical symptoms of this disease include dark rounded spots with a feathery edge on the adaxial side of the leaves while the abaxial epidermis remains unaffected. The disease can lead to the development of chlorosis

around the lesion and eventually defoliation (Blechert and Debener, 2005; Gachomo et al., 2006; Horst and Cloyd, 2007). Eleven unique races of *D. rosae* have been identified among the isolates obtained from North America and Europe (Whitaker et al., 2010b).

Two types of disease resistance to black spot have been characterized in roses. Vertical or complete resistance, which blocks sporulation and severely restricts the mycelial growth of the pathogen, is usually controlled by major dominant genes (*Rdr* or *Rosa* disease resistance genes) (von Malek and Debener, 1998; Whitaker et al., 2010a; Yokoya et al., 2000). In rose, the dominant resistance genes are pathogen race-specific, indicating a gene-for-gene interaction pattern (von Malek and Debener, 1998).

Partial or horizontal resistance that appears to be non-race-specific has also been identified in roses (Shupert, 2005; Whitaker and Hokanson, 2009a, 2009b; Xue and Davidson, 1998). This resistance does not prevent infection of the pathogen, but rather delays disease development and results in reduced lesion size, reduced sporulation, and/or delayed infection after inoculation (Parlevliet, 1981; Whitaker and Hokanson, 2009a, 2009b; Xue and Davidson, 1998). Compared with complete resistance, partial resistance is generally more durable over the range of pathogenic races (Noack, 2003). The ideal disease-resistant genotype should have both highly effective and long-lasting resistance to a broad spectrum of pathogenic races (Blechert and Debener, 2005), which can be achieved by pyramiding dominant complete resistance genes, obtaining strong partial resistance or by combining both types of resistances.

Black spot resistance is commonly evaluated in field trials in different geographic regions to expose the rose to a greater number of pathogenic races. These trials typically last 2–3 years to ensure sufficient disease pressure to properly assess the resistance of the plants (Carlson-Nilsson, 2002; Debener and Byrne, 2014; Noack, 2003; Shupert, 2005). The laboratory-based DLA is a tool for observing disease development efficiently under uniform and well-controlled environmental conditions and inoculum levels (Hattendorf et al., 2004; von Malek and Debener, 1998; Whitaker and Hokanson, 2009a, 2009b). Because single-conidial isolates are used in laboratory screening, the combination of compatible and incompatible interactions that are caused by various races in nature can be avoided (Blechert and Debener, 2005). However, as DLA only allows one cycle of disease development, the differences among genotypes might not be as accentuated as compared with a field trial in which multiple cycles of pathogen development are common (Xue and Davidson, 1998). Other factors such as the physical status of the host plant, degradation of the leaves, missing observations on leaf abscission/defoliation in DLA, and low or nonuniform inoculation levels in field assessment could all cause low correlation between these two methods of

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phenotyping (Johansson et al., 1992; Palmer et al., 1966; Zlesak et al., 2010).

The objectives of this research were to 1) evaluate two methods (DLA and WPI) of artificial inoculation for black spot disease evaluation and characterize rose genotypes for black spot resistance and 2) characterize partial black spot disease resistance derived from RW in laboratory tests in diploid populations to estimate the components of genetic variances, and the heritability of this partial resistance.

Materials and Methods

Plant materials. Based on previous field ratings for black spot resistance, seven black spot-susceptible roses ['Cal Poly' (Cal), 'Golden Gardens', 'Orange Honey' (OH), 'Red Fairy' (RF), 'Sweet Chariot' (SC), 'Vineyard Song' (VS), and 'Violette'], one with moderate resistance ('Old Blush'), seven black spot-resistant breeding lines (91/100-5, DD, FF, J06-20-14-3, J06-28-4-6, J06-30-3-6, M4-4, and one rose species RW) were propagated from cuttings and grown in 1-gal pots containing a growth media of decomposed pine bark amended with Metro-Mix growing media® (Sun Gro Horticulture Canada CM Ltd, Agawam, WA) under the greenhouse environment for 3 months before the experiments (Byrne et al., 2010; Zlesak et al., 2010). Nine individuals were randomly selected from each genotype for artificial inoculation for black spot resistance evaluation. The experiment was repeated three times.

Fifteen diploid populations were generated in an incomplete diallel mating design by crossing five black spot-resistant lines [J06-20-14-3 (J14-3), J06-28-4-6 (J4-6), J06-30-3-3 (J3-3), J06-30-3-6 (J3-6), M4-4] and a moderately resistant line ('Old Blush') with four susceptible roses ['Little Chief', RF, SC, and VS] to create F₁ populations segregating for black spot resistance (Table 1). The ploidy level of the parental lines was determined by chromosome counts. All the resistant lines have black spot resistance derived from RW. These were developed by crossing RW with either 'Old Blush' or 'Ducher' and then selecting among the F₂ and F₃ populations created by open pollination for high black spot resistance, recurrent bloom, and a high number of flowers. The moderately resistant and susceptible parents are commercial roses with excellent ornamental characteristics. All populations were maintained as either the original seedlings or clonal cuttings from the original seedling in 1-gal pots as previously described. At the age of 2 months, the plants were pruned back to synchronize shoot development to obtain shoots of similar physiological stage for inoculation. The pruning procedure was applied each time after collecting leaf samples.

Inoculation and data collection. Race 8 (also known as ACT) of *D. rosae*, originally collected in Brenham, TX, (Whitaker et al., 2010b) was selected as the pathogen race to use for the evaluation of partial resistance

because the parents in this project showed partial resistance but not complete resistance to race 8. Thus, they do not contain *Rdr3* vertical resistance gene to race 8. Conidia of race 8 of *D. rosae* (supplied by the Hokanson laboratory at the University of Minnesota) was collected by washing the infected leaves of 'Cl. Pinkie' with distilled (DI) water. The concentration of the conidia was adjusted to 1×10^5 conidia/mL with a hemocytometer (Whitaker and Hokanson, 2009a; Whitaker et al., 2010a).

Detached leaf assay. From each plant in an individual pot, up to seven fully expanded young compound leaves (fourth to sixth nodes from apex of each shoot) were collected for each replication. After washing with DI (distilled) water for 10 s on each side, the seven leaves were placed on wet paper towels in a transparent plastic container (152 × 140 × 59 mm). The conidia suspension (0.75 mL of 1×10^5 conidia/mL) was sprayed onto the leaves from a distance of 20–30 cm to generate fine mist, left for 48 h, and then residual water was removed by blotting. The droplet method was not used because it tended to cause leaf degradation. DI water was added to the paper towel without direct contact with the leaves to maintain the humidity in the boxes at 100%. The inoculated leaves were then maintained in the laboratory (≈25 °C and 10-h photoperiod) for 14–16 d and then inspected for the incidence of acervuli under the dissecting microscope. The LAS (percentage of leaf area with symptoms) and LL (diameter of the largest individual lesion) data were collected. Although other components such as incubation period, number of lesions, and sporulation capacity could all be used to characterize the disease resistance ability, LAS and LL were chosen here due to their high linear correlations and low-labor requirements which make them appropriate for massive screening of breeding materials (Xue and Davidson, 1998). All evaluations for the rose genotype comparison and the seedling evaluations were repeated three times.

Whole plant inoculation. Three plants of each genotype with branches of a similar size

were selected and sprayed with a conidial suspension (1×10^5 conidia/mL) until the leaf surface was completely wet. The wet tissue was then covered with a plastic bag that was sealed around the stem for 1 week. Additional DI water was sprayed into the bags for high-humidity (100%) maintenance. The inoculated plants were then maintained in the laboratory (≈25 °C, 10-h photoperiod with a humidifier) under cool white fluorescence lights (2150 lumens, Sylvania Fluorescent Light Bulb SL21371; Sylvania, Wilmington, MA). Four weeks after inoculation, acervuli incidence was checked under the dissecting microscope. LAS and LL data under WPI were collected on various leaves since the leaf size varied among breeding lines for similar biomass. Each plant generated one data point. The entire experiment was repeated three times.

Partial resistance was measured by the diameter of the largest individual lesion (LL) and the percentage of LAS. The visual rating score of the leaf area with lesions was as

Table 1. Parents², parental black spot resistance², and population size of the diploid rose populations used in the genetic study.

Female parent	Pollen parent	Population size
J14-3 (HR)	SC (S)	57
SC (S)	J14-3 (HR)	58
J14-3 (HR)	LC (S)	140
J14-3 (HR)	RF (S)	130
J14-3 (HR)	VS (S)	93
VS (S)	J14-3 (HR)	12
M4-4 (HR)	SC (S)	26
SC (S)	M4-4 (HR)	118
M4-4 (HR)	VS (S)	10
J4-6 (HR)	RF (S)	97
SC (S)	J4-6 (HR)	23
OB (MR)	J3-6 (HR)	112
OB (MR)	M4-4 (HR)	54
OB (MR)	RF (S)	158
J3-3 (HR)	RF (S)	38

²J14-3 = J06-20-14-3; J4-6 = J06-28-4-6; J3-3 = J06-30-3-3; J3-6 = J06-30-3-6; OB = 'Old Blush'; LC = 'Little Chief'; RF = 'Red Fairy'; SC = 'Sweet Chariot'; VS = 'Vineyard Song'.

³S = susceptible; MR = medium resistance; HR = high resistance.

Fig. 1. (A) Spores bearing acervuli on infected leaf surface of 'Cl. Pinkie'. (B) Diagrammatic representation of leaf area with symptoms of black spot disease at 1%, 5%, 10%, 25%, 50%, or 75% in detached leaf assay.

Table 2. Least square means of black spot leaf area with symptoms (LAS) and black spot lesion length (LL) for 16 rose genotypes after infection with race 8 of *Diplocarpon rosae* with the detached leaf assay (DLA) and whole plant inoculation (WPI).

Genotype ^y	Least square means ^z				
	DLA		NF	WPI	
	LAS	LL		LAS	LL
91/100-5	1.50 bcdef	2.00 abcde	0.00 bz	1.00 b	2.00 abc
Cal	1.98 abcde	2.33 abcd	0.00 b	1.11 b	4.06 a
DD	1.00 f	1.03 cde	0.22 ab	1.00 b	1.46 bc
FF	1.75 abcde	1.25 cde	0.56 ab	1.67 ab	1.43 bc
GG	2.08 abcd	2.50 abc	0.22 ab	1.89 ab	3.39 ab
J14-3	1.28 cdef	1.15 cde	0.56 ab	1.56 ab	1.56 bc
J4-6	1.08 f	0.49 e	0.00 b	1.00 b	0.70 c
J3-6	1.23 def	1.46 cde	0.83 a	1.75 ab	1.50 bc
M4-4	1.11 ef	0.86 cde	0.11 b	1.56 ab	2.11 abc
OB	1.47 cdef	1.81 bcde	0.22 ab	1.67 ab	2.83 abc
OH	2.46 ab	3.29 ab	0.78 a	2.44 a	2.58 abc
RW	1.46 cdef	0.51 de	0.00 b	1.17 b	1.02 c
RF	2.53 a	3.44 ab	0.40 ab	1.56 ab	2.44 abc
SC	2.49 ab	3.89 a	0.56 ab	1.89 ab	2.28 abc
VS	2.17 abc	2.50 abc	0.78 a	1.56 ab	2.06 abc
Vio	1.13 def	1.25 cde	0.50 ab	1.33 b	2.81 abc

^zLeast square means within the components connected by the same letter are not significantly different at $P = 0.05$, with Student-Newman Kuels adjustment for LAS and LL.

^yJ14-3 = J06-20-14-3; J4-6 = J06-28-4-6; J3-3 = J06-30-3-3; J3-6 = J06-30-3-6; RW = *Rosa wichuraiana* 'Basye's Thornless'; Cal = 'Cal Poly'; GG = 'Golden Garden'; Vio = 'Violette'; OB = 'Old Blush'; OH = 'Orange Honey'; RF = 'Red Fairy'; SC = 'Sweet Chariot'; VS = 'Vineyard Song'; NF = number of fallen leaves.

Table 3. Correlation coefficients relating the number of fallen leaves (NF), leaf area with symptoms (LAS), and black spot lesion length (LL) from whole plant inoculation (WPI) and LAS and black spot LL from the detached leaf assay (DLA).

		WPI		DLA	
		NF	LAS	LL	LAS
WPI	LAS	0.68**			
	LL	-0.08	0.27		
DLA	LAS	0.39	0.58*	0.46	
	LL	0.34	0.58*	0.56*	0.91***

*, **, ***Significant at $P < 0.05$, 0.01, and 0.001, respectively (15 df).

follows: 1 for 10%, 2 for 20%, 3 for 30%, 4 for 40%, and 5 for 50% and above for the screening of the parents that had diverse genetic backgrounds. For phenotyping the progenies of hybrid populations with similar genetic background, LAS scores were categorized as 1%, 5%, 10%, 25%, 50%, or 75% and above to detect the smaller differences of resistance among them (Fig. 1).

Statistical analysis. All the boxes in DLA revealed successful inoculation with infected leaves although occasionally not all leaves showed symptoms or were severely degraded. Data were only collected on the leaves that developed symptoms and were not severely degraded to ensure the data quality. The mean LAS and LL was calculated for each box and the single value was used in further analysis. The statistical analysis was conducted by using JMP software, Version 10, and SAS software 9.3 (SAS Institute Inc., Cary, NC, 1989–2010). The normality and skewness of the population data (original and square root transformed) was analyzed by Kolmogorov–Smirnov (K–S) test (Razali and Wah, 2011), which indicated that a square root transformation

improved normality and decreased skewness. Linear correlation of LL and LAS was estimated by Pearson correlation method.

Genetic variances were calculated using the restricted maximum likelihood (REML) method, assuming all factors from this unbalanced design as random effects (Dieters et al., 1995; Holland et al., 2003; Littell et al., 1996). Variances of parents were considered as additive variance (V_A) and variances of progeny (V_P) were considered as nonadditive variance (V_D) (Connor et al., 2005). Narrow-sense heritability (h^2) was estimated by the genetic variance from the REML model, where $h^2 = V_A/V_P$ (Hallauer et al., 2010).

Narrow-sense heritability was also estimated by an offspring–midparent regression (Connor et al., 2005), where $h^2 = b = \text{cov}(O, MP)/\text{cov}(MP)$ (Falconer and Mackay, 1996), i.e., the slope of the regression is then the estimation of heritability, with R^2 indicating the fitness of the regression.

Results

Phenotyping of parental materials. Spore-bearing acervuli were observed on all parental genotypes whether using the DLA or WPI method, indicating that complete resistance to race 8 of *D. rosae* did not exist among the selected rose genotypes. Using LL and LAS data, the most resistant genotypes as determined by field observations (RW, M4-4, and J4-6) were clearly distinguishable from the roses rated as most susceptible to *D. rosae* (RF, SC, Cal, VS, and OH) (Table 2). The best resolution among rose genotypes was with the LAS data, which was also able to separate other field-resistant roses (91/100-5, DD, J3-6) from the susceptible genotypes. LAS data indicated that the highest rating is 2.46 and 2.44 obtained from OH from DLA and WPI, respectively, either due to the

resistance ability from the breeding lines selected for this study or the pathogenesis ability of race 8. Although various resistant abilities could be revealed by different measurement components, the trending of susceptibility indicated by artificial inoculation is similar to what has been observed in the field. Since partial resistance is a continuous trait, clearly defining a germplasm as resistant or susceptible material might not be practical. The two measures of resistance, LAS and LL, are well correlated ($R = 0.91$ at $P < 0.0001$; Fig. 2). Genotypes with a higher percentage of LAS being covered with lesions showed longer LL, indicating either of these two traits could be used as an indicator of the host plant response to the pathogen.

When using the WPI to quantify the black spot resistance of the genotypes, it was found that the rose genotypes with higher resistance generally had lower LAS, LL, and number of fallen leaves (NF) when compared with the most susceptible rose genotypes, but these groups were not consistently different (Table 2). This would suggest that the DLA approach is the better method for quantifying the relative partial resistance of rose to black spot. The various measures of black spot, LAS and LL, are generally correlated between DLA and WPI (R ranging from 0.46 to 0.58). With the WPI approach, although NF was correlated with LL, LL and LAS were not correlated. LAS and LL data from DLA were well correlated (Table 3). The significant correlation between DLA and WPI, revealed by this study (R ranging from 0.56 to 0.58), is slightly weaker than the research conducted by Whitaker and Hokanson (2009a), which may be due to the different genetic background of the materials used for screening or inoculation methods such as growth conditions and the races of *D. rosae* that were selected.

Disease distribution of diploid populations. Based on the results of K–S normality test, the LL and LAS data normality improved and skewness generally decreased after a square root transformation. Thus, all subsequent statistical analyses were done with the transformed data, but it should be noted that the conclusions reached with the untransformed data and transformed data were not different.

Correlations among resistance assessments. The correlation of individual progenies' partial resistance to black spot race 8 measured by LAS and LL (square-root-transformed data) from the detached leaf tests is 0.3 ($P < 0.0001$) (Fig. 3). The correlation of these two components was much higher ($R = 0.9$) when estimating among resistant and susceptible parental materials, probably due to the smaller range of resistance among the seedlings vs. parents as measured by LL (0.5–3.0 mm vs. 0.1–7.14 mm).

Genetic variation and estimation of heritability of disease assessments using the DLA. In this incomplete diallel mating design, narrow-sense heritability (additive variance/phenotypic variance) was estimated to

Fig. 2. Correlation of leaf area with symptoms (LAS) and lesion length (LL) measurements of partial resistance after infection with race 8 of *Diplocarpon rosae* with the detached leaf assay method.

Fig. 3. Correlation of the partial resistance to black spot race 8 as measured by leaf area with symptoms (LAS) and lesion length (LL) in detached leaf assays among individual seedlings of 15 diploid rose progenies.

Fig. 4. The slope of offspring-midparent regressions estimates the narrow-sense heritability of 15 diploid populations measured by (A) leaf area with symptoms (LAS) and (B) lesion length (LL) from detached leaf assay inoculated by race 8 of *Diplocarpon rosae* with R^2 indicating the fitness of the regression. Original data were transformed using a square root.

be 0.28 and 0.43 for LAS and LL, respectively, indicating this partial resistance trait is moderately heritable. The parental variance for LAS and LL account for 24% and 34% of the variance, while the progeny variance for LAS and LL accounts for 61% and 45% of total genetic variance, respectively, indicating important nonadditive genetic effects. In contrast, the narrow-sense heritability estimation using the offspring-midparent regression approach was 0.86 and 0.74 for LAS and LL, respectively. The fitness of the regressions (R^2) of LAS and LL was calculated as 0.47 and 0.43, respectively, indicating a fairly good estimation of the midparent and offspring performances (Fig. 4).

Discussion

Artificial inoculation on parental germplasm. From this project, several cultivars (RF, Cal, SC, VS, and OH) were rated as very susceptible to black spot. Interestingly, the breeding line J3-6, which is derived from the wild species RW and has a high level of partial resistance to black spot, had greater defoliation under WPI than the other resistant lines (Table 2). In contrast, Cal, a susceptible material based on field observation, showed no defoliation under WPI. It is possible that different resistant mechanisms occurred in the host plants.

Artificial inoculation results indicated that DLA could distinguish the performance of the genotypes better than WPI, and the LL and LAS of DLA were well correlated as previously reported by Xue and Davidson (1998). Our results also confirm previous studies (Whitaker and Hokanson, 2009b) that with the DLA approach, it is easier to create a uniform humid environment and it requires less input of time and facilities as compared with the WPI. The DLA is more appropriate for the phenotyping of large populations and cultivar collections. However, as LAS and LL data generated from WPI were well correlated with LAS and LL data generated from DLA (Table 3), WPI could be used as a complementary characterization method to DLA for those genotypes whose leaves degraded easily.

Genetic variances calculated from the mixed model (both LAS and LL) indicated that the additive variances explained 24% to 34% of the total variances, which is lower than that explained by nonadditive variances (45% to 61%) (Table 4). In contrast, the midparent-progeny mean regression indicated that both measures were mainly additive in inheritance with heritability estimates of 0.74–0.86. The normal breeding approach for rose, a clonally propagated crop, is recurrent mass selection. Thus, selection is based on the phenotype of the individual seedlings with the best phenotypes being used as parents for the next generation. This is an efficient approach for traits with high narrow-sense heritability. The narrow-sense heritability estimates for LL and LAS ranged by estimation approach REML ($h^2 = 0.28$ –0.43) and midparent regression ($h^2 = 0.74$ –0.86),

Table 4. Variance component^z distribution (%)^y and estimated heritability^x of 15 diploid rose populations measured by square-root-transformed leaf area with symptoms (LAS) and lesion length (LL) from detached leaf assay inoculated by race 8 of *Diplocarpon rosae*.

Components	σ^2_A (%)	σ^2_D (%)	σ^2_P (%)	h^2
LAS	23.9	61.1	85.0	0.28
LL	33.9	45.3	79.2	0.43

^z σ^2_P = Phenotypic variances based on populations of individuals; σ^2_A = additive variances based on variances of parents; σ^2_D = nonadditive variances based on variance of progeny.

^yPercent of total variances = percentage of total genetic variances caused by σ^2_A , σ^2_D , and σ^2_P .

^xNarrow-sense heritability = ratio of additive genetic variance to total phenotypic variance; $h^2 = \sigma^2_A / \sigma^2_P$.

indicating this approach should be moderately to highly effective. Nevertheless, if the nonadditive genetic variance is as large as the additive genetic variance as indicated by the REML analysis, the progeny performance of specific parents should also be factored into the choice of parents in population creation. From a complete factorial mating design of partially resistant and susceptible roses conducted by Whitaker and Hokanson (2009a), within-family variances are much lower than that of between-family variances. Therefore, selection for certain families (generated from specific parental combinations) followed by backcrossing to the parents with more advanced ornamental traits was suggested for future breeding as an efficient selection/breeding approach.

Different results of narrow-sense heritability estimated from genetic variances and offspring-midparent regression might due to the structure of hybrid populations as the incomplete diallel mating design used in this study reduces the power of estimating genetic variances. In either case, the data indicate that it should be possible to develop a rose with stable and high resistance to black spot based on partial resistance derived from RW.

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