Published May, 2010

NOTE

Evaluation of a Clonal Propagation Protocol to Obtain Replicated Disease Data on Infection by *Colletotrichum truncatum* in *Lens culinaris*

Sally Vail* and Albert Vandenberg

Crop Development Centre, Univ. of Saskatchewan, Saskatoon, SK S7N 5A8, Canada. Received 4 May 2009. *Corresponding author (sally.vail@usask.ca).

ABSTRACT

Colletotrichum truncatum causes anthracnose, a major disease of lentil (*Lens culinaris* Medikus) on the Canadian prairies. Single-plant phenotyping of resistance is unreliable, thus the feasibility of using clonal propagation of individual lentil plants to generate replicated ratings was evaluated. Experiments revealed that highly resistant and highly susceptible lines had consistent disease reactions in cutting- and seedling-derived plants. However, the reaction of partially resistant lines differed and was attributed to differences in phenology between the treatments. When seedlings and replicated cuttings of an F_2 population were assessed for disease, it was found that segregation patterns of resistant to susceptible plants of the cuttings were consistent with the seedlings. It is proposed that the cutting method may be more dependable than using single-plant evaluation for this pathosystem.

Abbreviations: RILs, recombinant inbred lines.

Canadian production of lentil, *Lens culinaris* Medikus, accounts for more than 29% of world production (Food and Agriculture Organization of the United Nations, 2008), and with increasing interest in the crop it is expected that disease will continue to be a major barrier to yield and quality optimization. Breeding for resistance in lentil to anthracnose, caused by the pathogen *Colletotrichum truncatum* (Schwein.) Andrus & W. D. Moore is of the utmost importance for the Crop Development Centre, especially to the highly virulent race Ct0, for which resistance is extremely rare within the cultivated germplasm gene pool. Given warm, moist conditions, anthracnose can cause significant yield losses (Chongo and Bernier, 2000). Symptoms initially appear as tan-colored lesions on stems and leaves, which enlarge, resulting in leaf defoliation and shoot dieback (Chongo and Bernier, 1999). Selection of improved resistance and study of disease resistance genetics is preferred in early generations of segregating populations. However, consistency in phenotyping disease resistance to *C. truncatum* in lentil on the basis of rating reactions of a single plant is difficult, mainly because of false incompatible reactions and varying levels of susceptibility. This is due to the effect of microenvironment and other experimental variables, even when testing is conducted under controlled conditions in a greenhouse or growth chamber.

Detached leaf assays are commonly employed for phenotyping disease resistance in early generations of segregating populations

Published in Crop Sci. 50:926–932 (2010).
doi: 10.2135/cropsci2009.05.0239
Freely available online through the author-supported open-access option.
Published online 10 Mar. 2010.

in genetic studies. For example, detached leaves have been used to assess anthracnose resistance in common bean (*Phaseolus vulgaris* L.) (Tu, 1986) and chocolate spot in faba bean (*Vicia faba* L.) (Bouhassan et al., 2004). Inoculating detached leaves of various *Lens culinaris* lines with race Ct0 of *Colletotrichum truncatum* can differentiate resistance from susceptibility; however, this method cannot clearly discriminate partially resistant lines from highly resistant lines (Linder et al., unpublished data). Significant differences in defense responses between detached and attached leaf assays were found in *Arabidopsis thaliana* inoculated with *C. higginsianum* (Liu et al., 2007). Both salicylic acid– and ethylene-dependent pathways were required for resistance to *C. higginsianum* in *A. thaliana* in intact plants; detaching the leaves interfered with the ethylene pathway, resulting in false compatible reactions (Liu et al., 2007). Thus, obtaining disease resistance values on replicated whole plants is essential, especially for *Colletotrichum* pathosystems.

Recombinant inbred lines (RILs) are often used to obtain replicated phenotypic data for disease resistance in segregating populations for genetic studies. However, the development of RILs is demanding of both time and resources. Micropropagation has been useful in increasing production of F_2 seed in intra- and interspecific *Lens* population development (Fratini and Ruiz, 2008) and is often used in lentil breeding and genetic research programs for boosting population sizes by increasing the number of plants of a single genotype. As this method is relatively simple, it could be used to generate plants to obtain replicated disease data for a single F_2 genotype. This method would facilitate collection of both replicated F_2 disease reaction data and production of F_3 seed from a single plant, which would prove to be very useful in genetic studies. A similar method was used for testing resistance to *Ascochyta rabiei* in *Cicer arietinum* (Tar'an et al., 2007).

To date, the genetics of resistance to race Ct0 of *C. truncatum* have not been studied extensively, as resistance has not been found in the *L. culinaris* genepool. Race Ct0 resistance genes from *Lens ervoides* have been transferred to *L. culinaris* (Fiala et al., 2009). Investigation of the resulting interspecific population suggested resistance derived from *L. ervoides* accession L01-827A was controlled by two recessive genes. However, it is expected these results were skewed due to segregation distortion.

The objective of this study was to evaluate use of clonal propagation to generate replicated data for single plants for scoring resistance to *C. truncatum*. The method was first evaluated on a range of lines with varying levels of resistance, then on an F_2 population segregating for resistance to the pathogen. Phenological and plant architectural differences between plants grown from seeds vs. cuttings were also assessed on the F_2 population to determine what differences exist and to see if they are associated with disease resistance classification.

MATERIALS AND METHODS

Cutting Protocol

Cutting-derived plants were developed by first growing mother plants from single lentil seeds in an 8 h photoperiod for 6 wk (21°C days, 15°C nights) in a GR48 growth room (Conviron, Controlled Environments Limited, Winnipeg, MB). The purpose of the short photoperiod was to prolong the vegetative phase of the mother plants, allowing plants to grow more vegetative tissue so that subsequent cuttings could be taken. Cuttings were then taken from the first node below the youngest fully expanded leaf and treated with 100 mg of STIM-ROOT No.1 (Indole-3-butyric acid 0.1%) (Nu-Gro IP Inc., Woodstock, ON) before placing in a Jiffy-7 peat pellet (Jiffy Products, Shippagan, NB). Ten days after cutting, rooted plantlets were transplanted into Sunshine Germinating Mix 3 soil (Sun Gro Horticulture Canada Ltd., Vancouver, BC) and fertilized once every 2 wk with 100 mL of soluble all purpose Plant-Prod 20–20–20 fertilizer (4 g/L water) (Plant Products Co. Ltd., Nu-Gro IP Inc., Brantford, ON). Seven days after transplanting the cuttings, the photoperiod was changed from 8 to 18 h to induce normal development through the physiological phases.

Comparison of Propagation Methods for Disease Assessment across Lines

Eight lines with varying levels of resistance to *C. truncatum* were assessed. The *L. culinaris* lines evaluated were highly resistant LR59-81; partially resistant VIR 421, 3155S-5, 'Redberry', and 'Indianhead'; and susceptible 'Eston' and Yerli Kirmizi. One resistant *L. ervoides* accession (L01-827A) was included in the survey, as it is a source of resistance for LR59-81 to the highly virulent race Ct0 (Fiala et al., 2009). Comparisons were made between disease ratings of cutting- and seedling-derived plants grown from a single seed of the same line. Seeds were planted in 10-cm diameter pots at the same time cuttings were taken. The experiment used a randomized complete block design with four replications of each line generated by both seedlings and cuttings.

Inoculation with *C. truncatum* was performed about 1 mo after cuttings were taken. At inoculation, all lines of seedling-derived plants except 3155S-5 and 'Indianhead' were in the reproductive phase; 'Eston', LR59–81, and L01–827A were in full bloom; and VIR 421,Yerli Kirmizi, and 'Redberry' had buds but no open flowers. The lines 3155S-5 and LR59-81 were the only cutting-derived plants not in the reproductive phase on inoculation. Cutting-derived plants of 'Eston', VIR 421, Yerli Kirmizi, 'Redberry', and 'Indianhead' had buds but not flowers. L01-827A was the only line of cutting-derived plants in full bloom. On average, phenological development of plants propagated from seed was more advanced than plants propagated from cuttings.

Inoculation with *C. truncatum* isolate 95A8 (race Ct0) was conducted at both low (4×10^4 spores/mL) and high (5×10^5 spores/mL) concentrations. Spores were retrieved from cultures grown in 5-cm diameter Petri dishes with oatmeal agar (30 g blended quick oats [The Quaker Oats Company, Peterborough, ON] and 8.8 g agar [Difco, Becton, Dickinson and Company, Sparks, MD], 1 L deionized H_2O) for 7 d. Plants were inoculated with an airbrush sprayer (Badger Mini Spray Gun Set, Model 250.4, Badger Air-Brush Co., Franklin Park, IL) at 140 kPa with approximately 15 mL of suspension. For 24 h postinoculation, plants were incubated at 100% relative humidity.

Disease ratings were performed 20 d postinoculation for the percentage of the main stem covered in lesions, the percentage of leaves affected by disease (including defoliation due to disease) using the scale described by Horsfall and Barratt (1945), and the percentage of shoots that had died back due to disease. Data analyses for low and high spore concentrations were performed separately. Levene's test for heterogeneity of errors (Levene, 1960) was applied to factors across all components (stem lesions, leaf lesions, and shoot dieback). Heterogeneity in percentage shoot dieback across propagation method (cuttings or seedlings) was corrected by requesting multiple residuals in the Mixed procedure in SAS (SAS Institute, Cary, NC) for analyses at both high and low spore concentrations. Similarly, heterogeneity across lines at low spore concentration for the percentage stem lesions was corrected by using nonpooled residuals for testing significance of different factors. Correcting for heterogeneity across lines was not possible in any other analyses using either transformations or nonpooled residuals. The significance of fixed effects (lines and propagation method) was tested in the Mixed procedure in SAS with blocks being a random factor. Least square means, standard errors, and *p* values for differences between pairs of means were also calculated using the PDIFF option. In addition, Spearman correlations were performed for each of the traits across all lines on the basis of the individual values in SAS.

Evaluation for Disease Resistance of a Segregating Population

To compare propagation methods using a segregating F_2 population, 35 F_2 plants from the cross LR59-81 × 'Redberry', parental lines, and susceptible check 'Eston' were grown as mother plants from which eight cuttings per plant were taken. One week after cuttings were taken, seedlings of F_2s, parents, and checks were seeded into 50-cell trays (75 mL/cell with a 4-cm diameter). Each experimental block contained two trays, one with a cutting treatment of one of each of the 35 F_2 cutting-derived plants and five cutting-derived plants of each parental and check line, and the other tray with a different set of 35 seedling-derived F_2s and five seedling-derived plants of each parent and the susceptible check. The blocks were arranged in a randomized complete block design with four replications. The entire experiment was repeated once.

Before inoculation, the plant height, number of nodes, number of shoots, number of flowering nodes, and number of nodes with pods for plants derived from both cuttings and seedlings were recorded. These traits were analyzed as generalized linear models with normal distribution in the Genmod procedure in SAS. In addition, the proportion of reproductive plants for the three lines and across the F_2 population, comparing the two propagation methods in each block, was analyzed as a generalized linear model with a binomial distribution and logit link function.

Inoculation and disease rating procedures were the same as described above; however, a single concentration of *C. truncatum* was used (10^5 spores/mL) and disease rating occurred 14 d postinoculation. When disease scores were tested for heterogeneity of errors, the lines and population were found to be heterogeneous for all parameters assessed (stem lesions, leaf lesions, and shoot dieback). Propagation method (cuttings compared to seedlings) was heterogeneous for shoot dieback. Where possible, heterogeneity was corrected by grouping data by line using the Mixed procedure of SAS; however, heterogeneity could not be corrected for shoot dieback. The analysis of variance (ANOVA; first performed in the general linear model [GLM] procedure in SAS) of the checks only as well as the entire data set indicated no significant difference between the experiment replicates, thus data were grouped together for further analysis. 'Redberry', LR59–81, and the susceptible check 'Eston' were first analyzed for differences between seedlings and cuttings using the Mixed procedure in SAS. Least square means were determined in the Mixed procedure for the check and parental lines; *p* values for differences between pairs of means were also calculated using the PDIFF option. Least square means for each of the 35 replicated F_2 genotypes were determined in a separate analysis.

On the basis of a classification where resistant plants have less than 50% disease and susceptible plants have more than 51% disease, individual F_2s were categorized by disease scores, using the least square means for the cutting-derived replicated F_2s and raw percentage disease scores for the seedling-derived F_2s. Using Chi-square tests, seedling- and cutting-derived F_2s were tested for fit to Mendelian segregation ratios for resistance being controlled by a single dominant gene, by duplicate dominant epistasis, by duplicate recessive epistasis, and by dominant and recessive epistasis. Fit to the different models was assessed by comparing rejection or failure of rejection to each of the models for both cuttings and seedlings. Furthermore, the Genmod procedure using the binomial distribution in SAS was used to test for differences in segregation ratios between cutting and seedling F_2s, and the Poisson distribution in a generalized linear model in SAS was used to test differences in the frequency distribution between seedlings and cuttings.

Owing to the relatively small number of cutting-derived F_2s analyzed ($n = 35$), results from the seedling-derived plants were compared to a separate experiment where 104 F_2 individuals from the same population were assessed for disease using the cutting method to obtain replications. Multiple cuttings of individual F_2 plants were taken as described above at regular intervals and rooted, then transplanted, to generate experimental units. Transplanting, inoculating, and rating procedures were the same as used on the F_2 cuttings and seedlings described above except that two blocks were inoculated at once, and the experiment was repeated four times for a total of eight replications. Least-square means of raw disease ratings were calculated using the Mixed procedure in SAS.

RESULTS AND DISCUSSION

The ANOVA comparing propagation method for disease assessment across lines indicated significant differences between cutting- and seedling-derived plants with respect to both stem lesions ($p = 0.01$) and shoot dieback ($p < 0.01$) at the low inoculant concentration. However, these differences were not present in experimental units inoculated with the higher concentration of spores (Table 1). When inoculated with the high concentration, very few differences were observed between means of cuttings and seedlings on all lines. An exception was significantly less disease on leaves and less shoot dieback on cutting-derived plants of 3155S-5 ($p < 0.05$ and < 0.01, respectively; data not shown). At the low spore concentration, however, several significant differences across lines between cuttings and

Table 1. Factorial mixed model analysis of variance for propagation method (cutting- vs. seedling-derived plants) on lines of lentil (*Lens culinaris* Medikus) inoculated with *Colletotrichum truncatum,* assessed as the percentage of (i) the main stem covered in lesions, (ii) leaves affected by disease, and (iii) shoot dieback.

Spore concentration	Source of variation	Stem lesions				Leaf lesions				Shoot dieback			
		nDf†	dDf‡	*F* value	*P*	nDf	dDf	*F* value	*P*	nDf	dDf	*F* value	*P*
4×10^4 spores/mL	Line	7	14.4	138.78	<0.01	7	48	7.35	<0.01	7	47.3	7.66	<0.01
	Propagation method§	1	22.6	7.16	0.01	1	48	3.78	0.06	1	47.3	15.37	<0.01
	Line × propagation method	7	14.4	2.20	0.10	7	48	0.79	0.60	7	47.3	1.75	0.12
5×10^5 spores/mL	Line	7	46	7.32	<0.01	7	46	7.95	<0.01	7	45.4	5.64	<0.01
	Propagation method	1	46	1.42	0.24	1	46	0.15	0.70	1	45.5	2.39	0.13
	Line × propagation method	7	46	1.41	0.22	7	46	1.36	0.24	7	45.4	1.43	0.22

†Numerator degrees of freedom.

‡Denominator degrees of freedom.

§Comparing plants grown from seedlings or cuttings.

seedlings were evident (Table 2). 'Redberry' and 'Indianhead' differed significantly with respect to percentage stem lesions, with the cuttings having less disease. Furthermore, the percentage shoot dieback was significantly less on the cuttings of 3155S-5, 'Redberry', and 'Indianhead'. Correlations between seedling and cutting ratings were significant, with values of 0.62 ($p < 0.01$) for leaf lesion ratings, 0.65 ($p < 0.01$) for stem lesions, and 0.36 ($p < 0.05$) for percentage shoot dieback.

Both 'Redberry' and 3155S-5 have 'Indianhead' as part of their pedigree, which may explain the comparable differences in disease ratings of cuttings vs. seedlings. Resistance in 'Indianhead' declines as the plant begins to flower (Chongo and Bernier, 2000), and thus the differences observed in these lines may, in part, be owing to lack of synchronization of phenology between cutting- and seedling-derived plants. When the correlations of disease ratings between cuttings and seedlings inoculated with a low spore concentration are recalculated without these three lines, the values increase to 0.74 ($p < 0.01$) for stem lesions, 0.75 ($p < 0.01$) for leaf lesions, and 0.65 ($p < 0.01$) for shoot dieback.

When mother plants of the eight different lines were grown out at 18-h days postcutting, they produced 140 to 830 seeds per plant, which is sufficient seed to pursue further genetic studies in subsequent generations. Based on the results of the initial study, clonal propagation of single F_2 plants held promise as a method to obtain replicated disease evaluation data for *C. truncatum* resistance; however, reasons for the subtle differences in disease levels on some lines need to be explored fully.

In the segregating population, cutting- and seedling-derived plants differed significantly with respect to the number of shoots, the number of flowering nodes, and the number of nodes with pods (ANOVA; data not shown). More shoots were consistently observed on cuttings than seedlings on the basis of pairwise comparisons across the parental and check lines as well as on the F_2 population (Table 3). During the process of rooting cuttings, hormones in the plant may be produced that reduce the apical dominance exhibited by plants grown from seed. Cutting-derived plants significantly differed in the number of nodes compared with seedlings on each line; however,

Table 2. Mean disease ratings of the percentage of (i) the main stem covered with lesions, and (ii) shoot dieback due to infection with *Colletotrichum truncatum* on both cutting- and seedling-derived plants.

	Survey across lines (4×10^4 spores/mL)						Evaluation on parental lines and a susceptible check when inoculated with a segregating population					
	Stem lesions			Shoot dieback			Stem lesions			Shoot dieback		
Line	C†	S†	C vs. S‡	C	S	C vs. S	C	S	C vs. S	C	S	C vs. S
	%											
3155S-5	29.69	58.59	28.91 ±18.12	8.25	55.00	46.75 ±22.73*						
Yerli Kirmizi	94.28	98.62	4.34 ±4.25	100.00	100.00	0.00 ±22.73						
'Redberry'	25.00	81.34	56.34 ±18.88*	25.00	93.75	68.75 ±22.73**	95.35	71.26	24.09 ±4.68**	100.00	62.71	37.29 ±4.17**
LR59-81	5.86	6.44	0.59 ±4.40	0.00	25.00	25 ±22.73	2.60	2.47	0.14 ±0.15	0.00	0.06	0.06 ±4.29
'Eston'	98.62	94.28	4.34 ±4.18	100.00	100.00	0.00 ±22.73	98.10	96.51	1.60 ±0.11	100.00	99.48	0.52 ±4.20
L01-827A	22.65	21.09	1.56 ±14.34	24.00	59.50	35.50 ±22.73						
'Indianhead'	21.09	63.72	42.62 ±16.62*	0.00	74.00	74.00 ±22.73**						
VIR 421	58.68	55.86	2.83 ±30.49	56.25	58.25	2.00 ±22.73						
Mean	44.48	59.99	15.51 ±5.79*	39.19	70.69	31.50 ±8.04**						

*Significantly different from zero at $P < 0.05$.

**Significantly different from zero at $P < 0.01$.

†Mean of cutting- (C) or seedling-derived plants (S).

‡Difference in values between cutting- and seedling-derived plants, standard error of the mean, and significance level.

Table 3. Mean number of shoots, nodes, flowering nodes, nodes with pods, or height of cutting- or seedling-derived plants on three lentil (*Lens culinaris* Medikus) lines and a segregating F_2 population from the cross LR59-81 × 'Redberry'.

Line	Shoots			Nodes			Flowering nodes			Nodes with pods			Height		
	C†	S†	C vs. S‡	C	S	C vs. S	C	S	C vs. S	C	S	C vs. S	C	S	C vs. S
	no.														
LR59-81	5.4	1.8	3.6 ±0.4**	15.0	13.5	1.5 ±0.5**	5.5	3.4	2.1 ±0.5**	2.3	0.5	1.9 ±0.4**	30.0	28.5	1.6 ±2.4
'Eston'	6.5	1.6	4.9 ±0.4**	13.1	14.7	1.7 ±0.5**	5.6	2.7	2.9 ±0.5**	2.7	0.1	2.5 ±0.4**	26.9	36.4	9.5 ±2.3**
LR59-81 × 'Redberry' F_2 population	4.3	1.5	2.8 ±0.2**	14.5	14.0	0.5 ±0.2**	7.6	3.2	4.4 ±0.2**	4.1	0.4	3.7 ±0.1**	35.4	35.4	0.0 ±0.9
'Redberry'	4.8	1.5	3.3 ±0.4**	12.7	13.8	1.1 ±0.5*	6.9	1.3	5.5 ±0.5**	3.7	0.0	3.7 ±0.4**	30.2	30.3	0.1 ±2.3
Total	5.2	1.6	3.6 ±0.2**	13.8	14.0	0.2 ±0.2	6.4	2.7	3.7 ±0.2**	3.2	0.2	3.0 ±0.2**	30.6	32.6	2.0 ±1.0

*Significantly different from zero at $P < 0.05$.

**Significantly different from zero at $P < 0.01$.

†Mean of cutting- (C) or seedling-derived plants (S).

‡Difference in values between cutting- and seedling-derived plants, standard error of the mean, and significance level.

they varied as to which plant type had more. In contrast to the survey across lines, the results of this experiment suggest cuttings may have been phenologically older than seedlings, as each line had significantly more nodes with reproductive structures and nodes with pods. However, the number of plants in the reproductive phase was the same across the two treatments on the basis of analysis using the binomial distribution (data not shown). With the exception of 'Eston', cutting- and seedling-derived plants were not significantly different in height (Table 3).

Data for disease levels of cutting- and seedling-derived plants for the resistant parent LR59-81 and susceptible check 'Eston' were consistent with the initial experiment of this study, with no significant difference between cuttings and seedlings for all disease parameters assessed. Significant differences between disease levels on cuttings and seedlings were observed only on 'Redberry'; in contrast to the initial experiment, cuttings had significantly more disease than seedlings (Table 2). In the first experiment, differences seen in lines with 'Indianhead' in the pedigree were attributed to lack of growth phase synchronization. The results of the second experiment are consistent with phenological staging of the plants and support this assumption. In the *C. higginsianum–A. thaliana* pathosystems, detaching leaves triggered senescence pathways that interfere with disease response causing false compatible reactions (Liu et al., 2007). Not only may differences in the disease reaction observed on partially resistant lines have been due to differences in phenology but they have been enhanced by interference with the ethylene pathway when the cuttings were taken. When this method was proposed, the length of time between the initial propagation of the plant to the time of inoculation was presumed to be sufficient to restore these pathways to full capacity. Our results suggest the reaction of lines with partial resistance may be due to residual effects of the cutting procedure; however, the genetic tools to easily test this in *L. culinaris–C. truncatum* pathosystems do not currently exist.

Comparison of frequency distributions of disease scores of the F_2 genotypes using the Poisson distribution indicated significant differences between cuttings and seedlings (data not shown). A clear bimodal distribution was present for all parameters (stem lesions, leaf lesions, and shoot dieback) in the seedlings. However, the distinction between susceptible and resistant genotypes was not as clear in the cuttings; the lower end of the shoot dieback scale was skewed toward higher percentages in the cuttings compared with the seedlings (Table 4). Consistency in scoring of the percentage of leaves with lesions between cutting- and seedling-derived plants compared with the other disease parameters suggests

Table 4. Comparison of frequency distributions of seedling- and cutting-derived F_2s from the cross LR59-81 × 'Redberry' inoculated with *Colletotrichum truncatum* for percentage of (i) main stem covered with lesions, (ii) leaves affected by disease, and (iii) shoot dieback.

	Stem lesions				Leaf lesions				Shoot dieback			
	Count		Percentage†		Count		Percentage		Count		Percentage	
Disease (%)	S‡	C‡	S	C	S	C	S	C	S	C	S	C
0	0	0	0	0	0	0	0	0	174	5	66	14
1–25	200	17	75	49	163	7	62	20	18	9	7	26
26–50	8	8	3	23	25	8	9	23	28	9	11	26
51–75	8	1	3	2	20	11	8	31	3	3	1	9
76–100	49	9	18	26	57	9	22	26	42	9	16	26
Total	265	35	100	100	265	35	100	100	265	35	100	100
Average Disease§	24.44	40.51			36.27	54.22			22.02	41.84		

†The count as a percentage of the total number of F_2 genotypes.

‡Count or percentage of cutting- (C) or seedling-derived (S) plants.

§Average across all seedlings ($n = 265$) and of F_2s over 8 cutting replications ($n = 35$).

Table 5. Cutting- and seedling-derived F_2 population Chi-square test scores for genetic control of resistance ratios in *Lens culinaris* (LR59-81 × 'Redberry') inoculated with *Colletotrichum truncatum* for percentage of (i) the main stem covered in lesions, (ii) leaves affected by disease, and (iii) shoot dieback.

		Single dominant (3:1†)		Duplicate dominant (15:1)		Dominant and recessive (13:3)		Duplicate recessive (9:7)		
		S‡	C‡	S	C	S	C	S	C	P‡
	N§	265	35							104
Stem lesions	Ratio¶	208:57	25:10							76:28
	χ^{2}#	1.59	0.12	112.4	30.74	0.99	1.73	53.51	2.79	11.64
	P++	0.21	0.73	<0.01	<0.01	0.32	0.19	<0.01	0.09	<0.01
Leaf lesions	Ratio	188:77	23:12							31:73
	χ^{2}	2.17	1.27	248.86	48.55	17.02	4.73	23.42	0.98	27.92
	P	0.14	0.26	<0.01	<0.01	<0.01	<0.05	<0.01	0.32	<0.01
Shoot dieback	Ratio	220:45	24:11							71:33
	χ^{2}	8.77	0.54	56.22	39.14	0.63	3.04	77.44	1.77	5.87
	P	<0.01	0.46	<0.01	<0.01	0.43	0.08	<0.01	0.18	<0.05

†Ratio for genetic model being tested.

‡Results for cuttings (C), seedlings (S), or cuttings of the population (P) tested separately.

§Number of F_2 genotypes analyzed.

¶Resistant:Susceptible, with susceptible genotypes having ≤50% disease.

#χ^2 value of test for respective ratio.

++Probability of a greater χ^2 value under the null hypothesis of the ratio tested with Yates correction for continuity.

that this evaluation may more reliably predict resistance when the clonal propagation protocol is used (Table 1). However, this parameter is often skewed toward susceptibility in highly resistant lines (data not shown) and did not as clearly differentiate segregating population into susceptible and resistant classes as the other parameters assessed, especially for the cutting-derived F_2 genotypes (Table 4).

Binomial analysis comparing the number of F_2 genotypes classified as resistant or susceptible indicated a significant difference between cuttings and seedlings ($p < 0.01$) for all parameters assessed (data not shown). On the basis of the percentage of stem lesions of seedlings vs. cuttings from the same experiment, there was failure to reject models for genetic control by a single dominant gene (3R:1S) or by dominant and recessive epistasis (13R:3S), and the model for duplicate dominant epistasis (15R:1S) was consistently rejected ($P < 0.05$) (Table 5). Differences in Chi-square significance between cuttings and seedlings rated for shoot dieback assessed for fit to the single dominant gene model may be because of differences in original shoot numbers being assessed, as the cuttings tended to have more shoots than plants grown from seed (Table 3). On the basis of these differences and the lower correlation observed for this parameter in the initial experiment of this study, percentage shoot dieback due to disease should be interpreted with caution and not independently as an assessment of resistance when using the cutting method to generate replications. Discrepancies between data from cuttings and seedlings for the duplicate recessive epistasis model (9R:7S) were observed for all parameters (Table 5) and attributed to the small sample size ($n = 35$) for the cuttings. A Chi-square test on replicated F_2s ($n = 104$) from the same population, tested in a different experiment, rejected the null hypothesis of duplicate recessive epistatic control of resistance and was thus consistent with the seedling disease reactions observed in this experiment.

CONCLUSIONS

Clonal propagation of individual lentil plants is a feasible method for generating replicated ratings for reaction to infection by *Colletotrichum truncatum*. In our experiments, the seedling treatment resolved the population into more discrete classes in the frequency distributions, indicating the cutting method may distort the results. However, clear classes of resistant and susceptible genotypes are still evident with the cuttings (Table 4) for percentage stem lesions and percentage shoot dieback, and segregation of individuals using these parameters is consistent with reactions in seedlings of the same population. Results on both seedling- and cutting-derived F_2 ratings suggest the resistance to race Ct0 of *C. truncatum* in LR59-81 derived from *L. ervoides* is controlled by either a major single dominant gene or dominant and recessive epistasis.

Considering the interference reported in the disease-signaling pathway when detaching leaves in the *C. higginsianum* pathosystem on *A. thaliana* (Liu et al., 2007), the authors initiated these experiments, as they felt a detached leaf assay was not suitable for phenotyping resistance to *C. truncatum* in lentil. Furthermore, a previous pilot study testing a detached leaf assay for resistance to *C. truncatum* on lentil could only differentiate highly susceptible 'Eston' from partially resistant lines but did not reflect differences in resistance between highly resistant LR59-81 and partially resistant VIR 421 (Linder et al., unpublished data). Comparison of cutting-derived plants in the initial experiment of this study showed consistent significant differences between LR59-81 and VIR 421

(data not shown). Thus, although not ideal, this propagation method provides an attractive alternative by generating replicates from a single plant compared with using detached leaves to assess disease. Furthermore, the propagation method may also be able to reveal the complexity of a trait as it amplifies subtle differences between lines or genotypes.

Results of this study suggest disease evaluation may yield misleading results if shoot dieback on cuttings inoculated with *C. truncatum* is the only parameter assessed. The results from this particular F_2 population also suggest this method may be more appropriate in a population where age-dependent partial resistance is not involved. Even given this complexity, the results will still be useful when supported by the phenotypic characterization, if required, of the segregating F_3 families. The replication generated via the propagation protocol allows a cushion for failed inoculations that are common with this pathogen; replications are needed to overcome the variability introduced with larger experiments when testing larger population sizes. Furthermore, use of this method allows for development of F_3 families from each F_2 plant and subsequent development of RILs. Information from the earlier generation may indicate if further investment and continued development of the population is warranted to investigate complex traits. The ability to test specific F_3 families based on a replicated F_2 disease resistance phenotype and correlation of results between generations may prove to be a valuable tool in deciphering the genetics of this particular pathosystem.

Acknowledgments

Funding for this project was provided by the Saskatchewan Pulse Growers. We thank E. Linder for data on the detached leaf assay, S. Boechler for technical assistance, and G. Binsted for helpful feedback during manuscript preparation.

References

Bouhassan, A., M. Sadiki, and B. Tivoli. 2004. Evaluation of a collection of faba bean (*Vicia faba* L.) genotypes originating from the Maghreb for resistance to chocolate spot (*Botrytis fabae*) by assessment in the field and laboratory. Euphytica 135:55–62.

Chongo, G., and C.C. Bernier. 1999. Field and growth chamber evaluation of components of partial resistance to *Colletotrichum truncatum* in lentil. Can. J. Plant Pathol. 21:58–63.

Chongo, G., and C.C. Bernier. 2000. Effect of host, inoculum concentration, wetness duration, growth stage and temperature on anthracnose of lentil. Plant Dis. 84:544–548.

Fiala, J.V., A. Tullu, S. Banniza, G. Séguin-Swartz, and A. Vandenberg. 2009. Interspecies transfer of resistance to anthracnose in Lentil (*Lens culinaris* Medic.). Crop Sci. 49:825–830.

Food and Agriculture Organization of the United Nations. 2008. FAOSTAT. http://faostat.fao.org/site/567/default.aspx.

Fratini, R., and M.L. Ruiz. 2008. Micropropagation of intra and interspecific *Lens* hybrids. Euphytica 159:199–206.

Horsfall, J.G., and R.W. Barratt. 1945. An improved grading system for measuring plant diseases. Phytopathology 35:655.

Levene, H. 1960. Robust tests for the equality of variance. p. 278–292. *In* I. Olkin (ed.) Contributions to probability and statistics. Stanford Univ. Press, Palo Alto, CA

Liu, G., R. Kennedy, D.L. Greenshields, G. Peng, L. Forseille, G. Selvaraj, and Y. Wei. 2007. Detached and attached *Arabidopsis* leaf assays reveal distinctive defense responses against hemibiotrophic *Colletotrichum* spp. Mol. Plant Microbe Interact. 20:1308–1319.

Tar'an, B., T.D. Warkentin, A. Tullu, and A. Vandenberg. 2007. Genetic mapping of ascochyta blight resistance in chickpea (*Cicer arietinum* L.) using a simple sequence repeat linkage map. Genome 50:26–34.

Tu, J.C. 1986. A detached leaf technique for screening beans (*Phaseolus vulgaris* L.) in vitro against anthracnose (*Colletotrichum lindemuthianum*). Can. J. Plant Sci. 66:805–809.