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Evaluation of Detached Strawberry Leaves for Anthracnose Disease Severity Using Image Analysis and Visual Ratings

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Abstract. **Inoculation of detached strawberry leaves with *Colletotrichum* species may provide an accurate, rapid, nondestructive method of identifying anthracnose-resistant germplasm. The purpose of this study was to statistically compare two methods (visual and image analysis) of evaluating disease severity of strawberry germplasm screened for anthracnose resistance. Detached leaves of 77 susceptible and resistant strawberry clones were inoculated with one *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and two *C. fragariae* A. N. Brooks isolates. Anthracnose disease symptoms on each leaf were assessed quantitatively via computer-based image analysis to determine percentage lesion area and qualitatively by two independent raters using a visual disease severity rating scale (0 = no symptoms to 5 = entire leaf dead). The two visual raters' average disease severity ratings (n = 3413) were in substantial agreement with a weighted Cohen's kappa coefficient (*k*) of 0.80 [95% confidence interval (CI) 0.79–0.82]. There was a strong positive correlation between percent lesion area determined by image analysis and the visual disease scores of the two raters (r_p = 0.79). Image analysis provided a precise measurement of percent lesion area of infected leaves while visual assessment provided more rapid results. Our results indicate that detached leaf inoculations can be used as a rapid preliminary screen to separate anthracnose-susceptible from -resistant germplasm in large populations within breeding programs. It also may be used for assessing the resistance/susceptibility of parental breeding lines to various *Colletotrichum* species and isolates, for mapping germplasm for resistance genes, and in pesticide development studies.**

Anthracnose diseases on strawberry are destructive and may be caused by three *Colletotrichum* species: *C. acutatum* J. H. Simmonds, *C. fragariae*, and *C. gloeosporioides* (Maas, 1998; Smith, 1998). *C. acutatum* is the primary causal agent of anthracnose fruit rot and irregular leaf spot. Both *C. fragariae* and *C. gloeosporioides* may infect any above-ground part of the plant and incite anthracnose crown rot, anthracnose fruit rot, and anthracnose leaf spot, also called black leaf spot (Howard and Albregts, 1983). Warm temperatures and high humidity allow these pathogens to produce spores rapidly, and these are easily dispersed throughout production fields by rain splash, people, animals, insects, and equipment.

Anthracnose disease control strategies include planting disease-free plants, good sanitation, and the use of cultural and chemical controls; however, overuse of fungicides has resulted in pathogen resistance and failure of some fungicides to control anthracnose epidemics (Forcelini and Peres, 2018; LaMondia, 1995; Smith and Black, 1993a, 1993b). Ideal anthracnose disease control relies on the development and planting of disease-resistant cultivars. Many years are required to develop anthracnose-resistant strawberry germplasm with desirable plant growth habit, fruit taste and flavor, yield, and resistance to insects and other diseases. To identify disease-resistant germplasm, thousands of seedlings must be produced and evaluated for disease response in the field using natural inoculum or in greenhouse trials relying on artificial inoculation. However, inoculation trials are time-consuming, and many plants may be killed by the disease. This presents a problem for breeders because the plants killed in inoculation trials might have possessed other desired horticultural traits that could be used in their breeding program.

Screening strawberry germplasm for disease resistance using detached leaf assays is an alternative to inoculating whole plants that allows plant disease response to be determined without destroying the plant, reduces the time between inoculation and disease assessment, and confines the pathogen to the laboratory, which allows breeders to test for pathogens or races of pathogens from other geographic areas without transferring the pathogen to the field or risking its introduction to the industry. Inoculating detached leaves with a conidial suspension of *Colletotrichum* species was shown to be an accurate nondestructive method of identifying anthracnose-resistant germplasm by Miller-Butler et al. (2018).

Disease severity refers to the amount of plant tissue that is diseased (necrotic) and may be expressed as the percentage of plant area destroyed by a pathogen. Percentage or numerical disease assessment scales, such as visual rating scales, are often used for quick assessments of disease severity of plant tissue. Percentage scales often are adapted from the Horsfall-Barratt scale (Horsfall and Barratt, 1945), which contains 12 gradations, from 1% to 100%, with the percent disease varying disproportionately. Visual bias can influence accuracy, and percentage scales may be difficult to use when evaluating plants that exhibit noticeably different amounts of infection (James, 1977; Sherwood et al., 1983; Slopek, 1989). When there is little disease, the rater's visual focus is drawn to the small amount of necrotic or dark tissue compared with healthy or green tissue. When there is only a small amount of healthy tissue in a very diseased sample, the rater's visual focus is drawn to the small amount of healthy (green) tissue (Sherwood et al., 1983). Small areas of disease or no disease can be seen and a percentage can be determined fairly accurately, but when the lesion area ranges from 10% to 90%, it is much more difficult to give an accurate percentage. Slopek (1989) compared five variations of a 1 to 5 visual rating scale for estimating the percent diseased leaf area of barley plants and found that two of the five visual rating scales worked well for estimating the leaf disease, were as precise as the Horsfall-Barratt scale, and decreased

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Table 1. Average percent disease and rater average disease severity ratings (DSR) of detached strawberry leaves following inoculation with two *Colletotrichum fragariae* isolates (Cf63 and Cf75) and one *C. gloeosporioides* isolate Cg162. Approximately four leaves were inoculated with each of the three isolates in each of three trials.

	Colletotrichum spp.[z]				*C. fragariae*[y]				*C. gloeosporioides*			
Strawberry genotype	Disease (%)[x]		DSR[w]		Disease (%)		DSR		Disease (%)		DSR	
US70	0.5	u[v]	**0.2**[u]	c	0.5	p	**0.2**	f	0.5	lj	**0.3**	wx
US159	4.7	j-t	0.9	r-b	4.2	i-p	**0.8**	v-f	5.5	d-j	1.1	i-w
US292	5.3	i-u	0.9	q-a	5.0	h-p	1.1	l-d	5.6	d-j	**0.7**	l-x
MSUS478	7.3	g-u	0.9	n-z	7.2	d-p	**0.8**	t-f	7.3	b-j	1.1	g-w
MSUS518	2.0	r-u	**0.6**	x-c	2.9	l-p	**0.7**	x-f	0.5	ij	**0.5**	t-x
MSUS572	9.1	d-t	1.3	g-s	3.9	j-p	1.1	n-d	19.1	a-d	*1.7*	b-k
MSUS574	4.9	j-t	**0.8**	r-b	3.3	l-p	**0.7**	z-f	7.2	b-j	1.1	i-w
MSUS868	0.7	tu	**0.6**	x-c	0.8	op	**0.6**	a-f	0.6	ij	**0.5**	s-x
MSUS912	2.7	p-u	**0.7**	t-c	3.6	l-p	**0.7**	x-f	1.3	ij	**0.8**	l-x
MSUS922	6.9	g-u	1.1	j-w	3.9	j-p	0.9	q-f	12.4	a-j	1.5	b-n
MSUS927	4.1	n-u	0.9	r-b	5.6	g-p	1.2	k-c	1.0	ij	**0.4**	u-x
MSUS933	13.2	a-j	1.3	h-t	18.5	abc	1.4	h-y	4.1	e-i	1.1	i-w
MSUS944	7.7	e-u	**0.7**	v-c	11.4	c-o	**0.7**	z-f	1.5	ij	**0.7**	m-x
MSUS1010	2.6	p-u	**0.8**	s-b	2.5	m-p	**0.8**	v-f	2.7	g-j	**0.8**	l-x
MSUS1039	4.6	j-t	**0.7**	w-c	6.4	e-p	**0.8**	t-f	1.6	ij	**0.5**	r-x
MSUS1049	12.7	a-m	1.1	j-w	12.2	c-n	0.9	q-f	13.4	a-j	1.4	b-q
MSUS1055	8.4	d-u	**0.7**	t-c	9.8	c-p	**0.7**	y-f	5.7	d-j	**0.8**	i-x
MSUS1061	3.5	o-u	**0.7**	u-c	4.5	h-p	**0.7**	w-f	1.7	ij	**0.7**	i-x
MSUS1066	8.0	e-u	0.9	p-b	7.5	d-p	0.9	q-f	8.6	b-j	1.0	j-x
MSUS1078	6.7	g-u	0.9	o-a	3.0	l-p	**0.8**	v-f	12.7	a-j	1.2	f-v
MSUS1093	0.8	tu	**0.5**	y-c	1.0	op	**0.5**	c-f	0.4	ij	**0.5**	s-x
MSUS1094	6.7	g-u	0.9	r-b	9.6	c-p	1.0	o-e	1.7	ij	**0.7**	m-x
MSUS1105	11.8	a-n	*1.7*	b-j	12.8	d-m	*1.8*	b-m	10.1	a-j	1.5	b-o
MSUS1142	12.6	a-n	1.2	j-v	6.3	e-p	1.0	n-d	21.7	ab	1.4	b-r
MSUS1154	3.9	o-u	**0.5**	y-c	5.3	h-p	**0.6**	a-f	0.2	j	**0.1**	x
MSUS1180	0.8	tu	**0.4**	bc	1.0	op	**0.4**	def	0.3	ij	**0.3**	vwx
MSUS1196	6.2	i-u	1.0	k-w	4.6	h-p	0.9	p-e	9.1	b-j	1.1	g-w
MSUS1197	4.4	l-u	0.9	r-b	5.9	f-p	1.0	p-e	1.4	ij	**0.6**	o-x
MSUS1217	4.4	l-u	1.0	k-w	6.1	f-p	1.3	i-a	2.1	hij	**0.8**	l-x
MSUS1229	3.7	o-u	**0.8**	r-b	4.5	h-p	0.9	q-e	2.3	hij	**0.7**	n-x
MSUS1230	10.3	d-q	1.2	i-u	11.2	c-p	1.3	i-a	8.4	b-j	1.2	f-v
MSUS1240	10.8	a-p	1.3	h-t	12.2	c-n	1.3	i-a	9.0	b-j	1.3	f-u
MSUS1265	6.4	g-u	**0.8**	r-b	8.5	c-p	0.9	p-e	1.3	ij	**0.6**	q-x
MSUS1269	3.0	p-u	**0.7**	v-c	3.1	l-p	**0.6**	a-f	2.8	g-j	**0.8**	l-x
MSUS1270	4.3	m-u	**0.8**	r-b	5.6	g-p	0.9	p-e	2.2	hij	**0.6**	p-x
MSUS1271	1.8	r-u	**0.6**	w-c	1.4	nop	**0.8**	v-f	2.5	g-j	**0.4**	vwx
MSUS1311	8.1	e-u	0.9	r-b	7.2	d-p	**0.8**	t-f	9.6	a-j	1.0	j-x
MSUS1343	2.4	p-u	**0.5**	z-c	0.7	op	**0.3**	ef	5.4	d-j	**0.7**	l-x
MSUS1352	1.5	r-u	**0.4**	abc	1.8	nop	**0.4**	def	1.1	ij	**0.4**	t-x
MSUS1356	4.1	n-u	0.9	r-b	5.8	g-p	1.1	k-c	0.7	ij	**0.5**	r-x
MSUS1359	4.2	m-u	**0.7**	u-c	5.6	g-p	0.9	q-e	1.9	hij	**0.4**	t-x
MSUS1362	1.4	stu	**0.6**	x-c	1.6	nop	**0.5**	b-f	1.0	ij	**0.7**	m-x
MSUS1365	8.3	d-u	1.3	g-s	8.4	c-p	1.5	f-u	8.2	b-j	0.9	j-x
MSUS1426	3.0	p-u	**0.8**	s-b	3.4	l-p	0.9	q-f	2.3	hij	**0.6**	q-x
MSUS1432	3.3	o-u	**0.8**	t-c	4.4	h-p	0.9	r-f	0.5	ij	**0.5**	s-x
MSUS1511	9.4	d-s	0.9	r-b	4.8	h-p	**0.6**	a-f	18.6	a-e	1.4	b-p
Aiko	10.1	d-q	*1.9*	a-e	9.4	c-p	1.5	f-t	10.8	a-j	*2.3*	ab
Albion	11.7	a-n	*1.8*	a-h	13.8	b-k	*1.7*	c-o	8.7	b-j	*1.9*	a-i
Allstar	9.9	c-r	1.4	e-p	9.0	c-p	1.6	e-q	11.6	a-j	1.2	f-u
Aromas	7.4	f-u	*2.0*	a-d	7.8	c-p	*2.0*	a-h	6.8	d-j	*2.0*	a-f
Camino Real	13.4	a-i	*2.3*	a	16.3	a-g	*2.5*	a	9.2	b-j	*2.0*	a-h
Chandler	13.0	a-l	*1.8*	a-g	14.6	a-j	*1.9*	a-i	10.7	a-j	*1.7*	b-k
Diamante	10.0	c-r	1.6	c-k	16.3	a-g	*1.8*	a-j	1.3	ij	1.4	c-q
Dover	7.1	g-u	1.3	g-r	11.2	c-p	0.9	r-f	3.6	f-j	*1.7*	b-k
Earliglow	8.5	d-u	1.2	i-u	10.2	c-p	1.3	h-z	5.8	d-j	1.1	i-w
Elsanta	10.6	b-o	*1.7*	b-j	5.3	i-p	1.2	i-a	18.4	a-e	*2.3*	abc
Gaviota	11.9	a-n	*2.1*	abc	14.9	a-j	*2.5*	ab	7.4	b-j	1.5	b-p
Honeoye	16.8	a-d	*2.3*	a	17.0	a-e	*2.3*	abc	16.5	a-h	*2.1*	a-d
Jewel	12.9	a-l	*1.9*	a-f	13.7	b-l	*1.9*	a-j	11.6	a-j	*2.0*	a-g
Kent	19.2	a	*1.8*	a-g	24.7	a	*2.3*	a-d	11.2	a-j	1.1	g-w
Ovation	6.7	g-u	1.6	c-l	6.5	e-p	1.5	e-r	7.0	d-j	1.6	b-k
Pelican	3.0	p-u	**0.5**	y-c	3.2	l-p	**0.5**	def	2.7	g-j	**0.5**	q-x
Portola	11.6	a-n	*1.7*	b-j	16.7	a-f	*1.7*	c-n	4.7	d-j	1.6	b-k
Redchief	8.8	d-u	1.2	j-w	6.7	e-p	1.2	j-b	13.1	a-j	1.0	i-w
Salinas	12.7	a-m	*1.9*	a-f	4.6	i-p	1.4	g-x	23.9	a	*2.6*	a
Scott	18.0	abc	*2.2*	ab	13.8	a-j	*2.2*	a-e	24.2	a	*2.0*	a-f
Seascape	14.9	a-g	1.6	c-k	13.0	d-m	*1.7*	c-m	17.5	a-f	1.4	c-r
Selva	18.9	ab	*1.8*	a-i	24.1	ab	*2.1*	a-g	10.6	a-j	1.2	f-u
Senga Sengana	14.0	a-h	1.5	d-m	13.4	b-m	1.4	h-y	14.9	a-i	*1.7*	b-j
Sequoia	14.3	a-h	*1.9*	a-f	18.0	a-d	*2.2*	a-f	8.8	b-j	1.4	b-q
Strawberry Festival	4.0	o-u	1.5	d-m	5.3	i-p	1.4	g-v	2.1	hij	1.6	b-k

(*Continued on next page*)

Table 1. (*Continued*) Average percent disease and rater average disease severity ratings (DSR) of detached strawberry leaves following inoculation with two *Colletotrichum fragariae* isolates (Cf63 and Cf75) and one *C. gloeosporioides* isolate Cg162. Approximately four leaves were inoculated with each of the three isolates in each of three trials.

	Colletotrichum spp.[z]				*C. fragariae*[y]				*C. gloeosporioides*			
Strawberry genotype	Disease (%)[x]		DSR[w]		Disease (%)		DSR		Disease (%)		DSR	
Sweet Charlie	4.5	k-u	1.5	d-n	5.5	g-p	1.4	g-x	3.2	f-j	1.6	b-k
Surecrop	6.2	i-u	1.4	f-q	6.7	e-p	1.4	g-w	5.4	d-j	1.3	d-s
Tangi	15.9	a-f	1.6	c-k	12.2	c-n	1.6	d-p	20.7	abc	1.6	b-m
Tillamook	16.2	a-e	1.5	d-o	15.3	a-h	*1.9*	a-j	17.1	a-g	1.1	g-w
Tioga	7.6	f-u	*1.8*	a-h	5.9	f-p	1.5	f-s	10.0	a-j	*2.1*	a-e
Treasure	4.5	k-u	1.4	f-q	5.4	i-p	1.2	k-c	3.3	f-j	*1.7*	b-k
LSD	8.5		0.5		10.9		0.7		14.6		0.9	

[z]Average DSR and percent disease for all three *Colletotrichum* spp. isolates combined.
[y]Average DSR and percent disease for two *Colletotrichum fragariae* isolates, Cf63 and Cf75, combined.
[x]Percent disease was calculated from computer image analysis of photographed leaves.
[w]Average DSRs are based on the average of two visual raters' DSR.
[v]Means within columns followed by different letters are significantly different according to Fisher's protected least significant difference (LSD; $P < 0.05$).
[u]DSRs in bold print indicate a resistant anthracnose rating, and those in italics indicate a susceptible anthracnose rating based on detached leaf resistance categories reported by Miller-Butler et al. (2018): DSR ≤ 0.8 = resistant; >0.8 to ≤1.6 = intermediate; and >1.6 = susceptible.

Table 2. Frequency of the two visual raters' disease severity ratings (DSRs) within each level of the 0 to 5 visual rating scale for symptoms on 3413 detached strawberry leaves inoculated with *Colletotrichum* spp.[z]

Rater 1	Rater 2 DSR					
DSR[y]	0	1	2	3	4	5
0	1015	792	2	0	0	0
1	43	550	10	0	0	0
2	3	320	42	0	0	0
3	0	113	135	19	2	0
4	0	9	65	111	37	3
5	0	0	0	11	32	99

[z]Two isolates of *Colletotrichum fragariae* (Cf63 and Cf75) and one isolate of *C. gloeosporioides* (Cg162) were used to inoculate the detached strawberry leaves. Each isolate was applied separately to a different leaf.
[y]The rater average DSRs are based on the average of two visual raters' DSR.

the time required for disease assessment. Other researchers have suggested the use of an equal interval scale over the Horsfall-Barratt scale for assessing disease severity (Nita et al., 2003) or the use of illustrated assessment keys with standard area diagrams of percentage scales that allows standardization in disease assessment methods for a variety of crops (James, 1977).

Disease symptoms on plants or leaves are often evaluated using numbered grade scales, sometimes referred to as arbitrary, nominal, or ordinal scales, which have some degree of subjective interpretation of the disease by the rater. An ordinal scale of 0 to 5 (0 = no disease, 1 = very slight, 2 = slight, 3 = moderate, 4 = severe, 5 = dead plant) is only interpretable in the arrangement of the order and can only provide qualitative data. Many disease assessment keys are ordinal and cannot quantitatively measure a difference between the values but were satisfactory when used by experienced observers for rating plants or plots in an order of increasing symptom severity (Russell, 1978).

When there is more than one visual rater, good agreement and association between raters are desirable. Understanding the interpretation of results from ordinal data can be explained by the concepts of accuracy (agreement) and precision (association). Accuracy is the raters' ability to rate disease closest to a true value (such as percent disease measurements determined by image analysis) and precision is the repeatability of the scoring; however, accuracy and precision may not coincide. Statistical analysis helps determine whether raters are interpreting disease the same or very close to the same. The Kappa coefficient (k) (Cohen, 1960) is a measure of the difference between the raters' agreement and the expected agreement. It determines a proportion of agreement (accuracy), correcting for chance agreement and is scaled to vary from –1 to +1. A negative Kappa coefficient indicates less than chance agreement, zero indicates exactly chance agreement, a positive value indicates better than chance agreement, and +1 indicates perfect agreement. The Kappa coefficient has been interpreted as <0 = less than chance agreement, 0.01 to 0.20 = slight agreement, 0.21 to 0.40 = fair agreement, 0.41 to 0.60 = moderate agreement, 0.61 to 0.80 = substantial agreement, and 0.81 to 0.99 = almost perfect agreement (Viera and Garrett, 2005). Agreement and disagreement are not mutually exclusive in an ordinal rating scale. If two raters see the disease on a plant as slight disease and moderate disease, they are not in complete agreement, but they are not in complete disagreement either because both raters have determined that there is disease on the plant. This problem was addressed with the weighted Kappa coefficient by assigning weights to different degrees of disagreement with lesser weight to agreement because categories are further apart (Cohen, 1960, 1968; Fleiss and Cohen, 1973). The degree of linear association (precision) between two sets of data, such as the two visual raters' disease severity ratings, can be statistically measured with Pearson's product moment correlation coefficient (r_p). A positive Pearson correlation coefficient designates that both sets of data change in the same direction, and a negative Pearson correlation coefficient designates that both sets of data change in opposite directions.

Precise quantitative analysis can be performed on images of diseased plant tissues using computer software. Electronic images can be stored indefinitely, allowing the researcher to process the images as time permits. Digital imaging and analytical software were used by Wang et al. (2008) to develop a miniaturized strawberry leaf disk antifungal bioassay. Their goal was to determine percent disease caused by a *Colletotrichum* isolate used for inoculation of a leaf disk, as well as the percent phytotoxicity that might have been caused by the antifungal compounds using 15-mm excised strawberry leaf disks. The leaf disks were dipped in antifungal compounds and then inoculated with the same *Colletotrichum* species being used in this research. The analyzing software transformed the images to show healthy parts of the leaf as green, diseased parts as black, and parts exhibiting phytotoxicity as gray. In a similar study (Abril et al., 2009), photographs of detached strawberry leaves were used for visual assessment of disease severity in a study testing the efficacy of natural product-based fungicides, and the percent diseased leaf area was assessed with an arbitrary scale of 0 = no disease to 3 = most severe disease. When digital image analysis and visual assessment were both used by Kwack et al. (2005) to determine the percentage of diseased area caused by *C. orbiculare* on cucumber leaves, visual ratings were significantly higher than the image analysis ratings, and the authors reported processing the digital images took longer. When rating severity of foliar citrus canker symptoms, Bock et al. (2009) also reported image analysis was slower compared with visual assessment; however, image analysis gave more precise and accurate results. In contrast, Nutter et al. (1993) reported image analysis as a measure of disease severity was faster compared with visual assessments.

The objective of this study was to compare visual assessments with image analysis of anthracnose disease on inoculated detached strawberry leaves to determine the degree of agreement and association between the two methods. This research expands on preliminary trials to develop a reliable detached leaf assay for anthracnose resistance in strawberry plants (Miller-Butler et al., 2013).

Materials and Methods

Plant material and fungal isolates. Thirty-one cultivars [purchased from commercial strawberry nurseries or obtained from the U.S. Department of Agriculture or the Agricultural Research Service (USDA-ARS), Germplasm Repository, Corvallis, OR] and 46 anthracnose-resistant selections (US and MSUS clones, Miller-Butler et al., 2018, 2019) maintained at the USDA-ARS, Thad Cochran Southern Horticultural Laboratory, Poplarville, MS (Table 1) were established in 10-cm plastic pots in a 1:1 mixture of sand and Jiffy-Mix (Jiffy Products of America Inc., West Chicago, IL), grown in a greenhouse at 28 °C ± 10 °C with a 16-h photoperiod, and screened for anthracnose resistance using a detached leaf assay.

Two isolates of *C. fragariae* (= *C. theobromicola*), Cf63 and Cf75, and one isolate of *C. gloeosporioides* (= *C. fructicola*), Cg162, were used as inoculum (Chang and Smith, 2007; Miller-Butler et al., 2018; Munir et al., 2016; Smith and Black, 1990). Each isolate was initiated from silica gel cultures and grown in petri dishes on oatmeal agar:potato dextrose agar (1:1) (Miller-Butler et al., 2018) under fluorescent lights with a 12-h photoperiod at a temperature of 20 to 28 °C. Inoculum was prepared as a conidial suspension from 7- to 14-d-old cultures by flooding each culture plate with sterile deionized water, gently scraping the agar surface with a glass rod to remove conidia, filtering the suspension through sterile cotton bandage gauze with 1 mm^2 openings (Johnson & Johnson, New Brunswick, NJ), and adjusting to a concentration of 1.5×10^6 conidia/mL by diluting with sterile deionized water containing one drop of the surfactant Tween-20 (Sigma Chemical Co., St. Louis, MO) per 1 L of water.

Detached leaf inoculations. Following the protocol reported by Miller-Butler et al. (2018), young, fully developed, blemish-free leaves were removed from plants no more than 4 h before inoculation and rinsed in tap water; the petiole of each leaf was then inserted into sterile deionized water in a 10 × 150 mm test tube. Detached leaves from each of the 77 clones were inoculated separately with a conidial suspension of each of three isolates, *C. fragariae* (isolates Cf63 and Cf75) and *C. gloeosporioides* (isolate Cg162) by misting the adaxial surface of the leaf blades with a hand-pump sprayer to the point of runoff. Each isolate was applied to a different leaf. Detached leaves used for noninoculated controls were misted with deionized water. Inoculated and noninoculated leaves were immediately placed in a dew chamber at 100% relative humidity (RH) and 30 °C, incubated in the dark for 48 h, and transferred to sealed, clear plastic containers at 100% RH and 28 °C with continuous fluorescent light for an additional 3 d before assessing disease symptoms. Disease symptoms on the blade of each leaf were visually assessed by two independent, trained raters using a disease severity rating (DSR) scale of 0 = no visible disease symptom on any leaflet, 1 through 4 = increasing disease symptom severity, and 5 = total area of leaflets necrotic (Miller-Butler et al., 2018).

Immediately after visual assessment, each leaf blade was separated into individual leaflets, placed on a light box, and photographed either with a DXCB151A color video camera (Hitachi Instruments, Inc., Houston, TX) and captured with Bioquant 98 image analysis software (R&M Biometrics, Inc., Nashville, TN) (trial 1) or with a Nikon COOLPIX 5000 digital camera (Nikon Corp., Tokyo, Japan) and uploaded as JPG files (trials 2 and 3). Photographs were enlarged electronically 200% and individually marked and colorized for image analysis with healthy leaf tissue as green and tissue with lesions as black using Corel Photo-Paint X4 or X5 (Corel Corp., Ottawa, Ontario, Canada). Lesions were counted electronically, but due to the nature of anthracnose

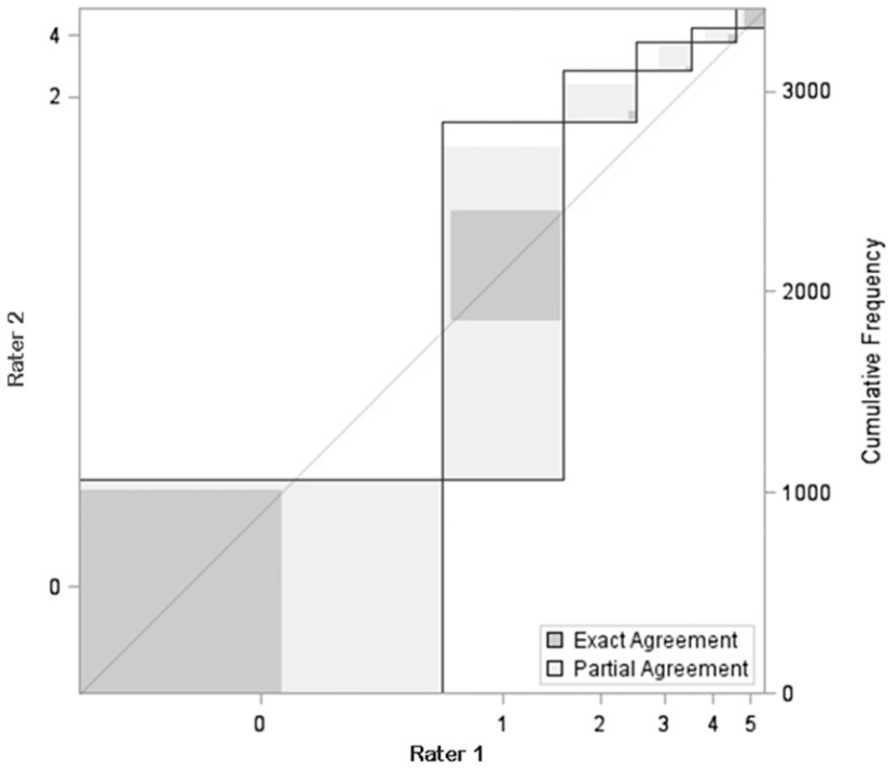

Fig. 1. Agreement between the two visual raters' disease severity ratings (k_w = 0.80, 95% confidence interval 0.79–0.82) of detached strawberry leaves following inoculation with three isolates of *Colletotrichum* spp. was better at the lower (0 and 1) and the higher (5) ends of a 0 to 5 scale. Ratings of 2, 3, and 4 had minimal perfect agreement.

Table 3. Summary statistics for average percent disease of 3413 detached strawberry leaves after inoculation with three isolates of *Colletotrichum*[z] corresponding to the raters' average disease severity rating (DSR) on the same leaves. Percent disease was calculated from computer image analysis of photographed leaves and averaged across the three isolates.

	Disease (%)					
Raters' DSR[y]	Avg	SD	Median	Minimum	Maximum	N
0	0.3	0.4	0.1	0.0	3.5	1309
1	1.2	1.5	0.7	0.0	15.3	1166
2	5.1	4.0	4.0	0.1	29.4	488
3	16.9	8.7	15.8	2.2	48.6	204
4	45.5	20.8	45.5	8.6	91.9	137
5	93.7	10.6	98.9	44.6	100.0	109

[z]Two isolates of *Colletotrichum fragariae* and one isolate of *C. gloeosporioides* were used to inoculate the detached strawberry leaves. Each isolate was applied separately to a different leaf.
[y]The rater average DSRs are based on the average of two visual raters' DSR.

symptoms on strawberry leaf blades (small lesions coalesce into a larger lesion as the disease progresses), these data were not used in separate analyses but were used to determine lesion area. Total leaf area was calculated as the green (healthy) area plus the black (lesion) area using Image Pro Plus 7.0 (Media Cybernetics, Bethesda, MD). Percent lesion area was calculated as lesion area divided by total leaf area multiplied by 100. The percent lesion area determined by image analysis was paired with the two visual raters' DSR, and both the image analysis and each rater's DSR were averaged for each clone across all repetitions for each isolate. Hereafter the electronic disease ratings determined by image analysis is termed *percent disease*.

Statistical Analysis. Detached strawberry leaves from each of 77 clones were inoculated with each of three *Colletotrichum* isolates, separately, in three trials conducted over 2 years. The availability of leaves at each collection date determined the number of leaves inoculated, but generally four leaves (replications) were inoculated and an average of 12 leaves of each clone were inoculated with each of the *Colletotrichum* isolates in the three trials. The experimental design was a completely randomized design. Association and agreement between the two independent raters were determined using Pearson's product-moment correlation coefficient (r_p) and weighted Kappa coefficient (k_w), respectively. Both raters' visual DSRs were then averaged together (rater average) and tested for association and agreement with the percent disease. The rater average for each number on the 0 to 5 rating scale was rounded to whole numbers. Percent disease and visual DSR were subjected to analysis of variance, and means were separated by Fisher's protected least significant difference method at $P < 0.05$ using SAS statistical software (version 8.2; SAS Institute, Cary, NC).

Results

A total of 4028 observations were made with an overall average of four leaves per clone per isolate per trial plus control leaves inoculated with deionized water at each inoculation date. The control leaves (615) had an overall average DSR for both raters of 0.09 and a percent disease of 0.40%, indicating that the detached leaves used at the inoculation date did not have disease symptoms before inoculation with *Colletotrichum.* Therefore, data from the controls were removed from further statistical evaluations.

The average DSRs of the two visual raters were in substantial agreement (Table 2) with a weighted Kappa of 0.80 (95% CI 0.79–0.82) (Fig. 1). There was perfect agreement between the two visual raters for 52% of the DSRs; i.e., perfect agreement 30% of the time for the DSR of 0, 16% perfect agreement for the DSR of 1, 1% agreement for the DSRs of 2, 3, and 4, and 3% perfect agreement for the DSR of 5. The two raters' DSRs of 2, 3, and 4 were not in total disagreement. Rater 1 rated 365 inoculated leaves as a 2, 269 as a 3, and 222 as a 4, whereas rater 2 rated 320 leaves as a 1, 135 as a 2, and 111 as a 3. The DSRs that were more than one disease rating from each other (e.g., one rater rated a leaf as a 2, and the other rated the same leaf as a 0) only totaled 203, or 6% of the 3413 DSRs, with most of this variation occurring for DSRs of 3 (113) and 4 (74). The association of the two raters' average DSRs was good with a Pearson's product-moment correlation coefficient of $r_p = 0.84$, and their agreement was substantial with a weighted Kappa of $k_w = 0.80$. The substantial agreement and good association between the two raters' DSRs showed consistency between the visual raters. Therefore, the DSRs of the two raters were averaged and considered the raters' average DSR used to compare with the image analysis ratings.

The association of the raters' average DSR (Table 3, Fig. 2) and the percent disease was good with a Pearson's product-moment correlation coefficient of $r_p = 0.79$. The weighted Kappa statistic determines agreement between two sets of data and requires both sets to contain the same number of scoring values. Due to the preciseness of the percent disease producing hundreds of percentage measurement values and the visual rating scale containing six measurement values, a weighted Kappa could not be calculated using the raters' average DSR and percent disease. An average raters' DSR of 0 was found to be equivalent to 0.3% disease, and the DSR of 5 was found to be equivalent to 93.7% disease. The percent disease separates well with the visual rater 0 to 5 DSR scale (Table 3, Fig. 2).

The average percent disease and raters' average DSR were determined for each of three fungal isolates used for the inoculations. The most aggressive of the three isolates was *C. fragariae* isolate Cf75 with an overall average percent disease of 10% and raters' average DSR of 1.4; *C. gloeosporioides* isolate Cg162 was the next most aggressive with ratings of 7.1% disease and 1.1 average DSR; and *C. fragariae* isolate Cf63 was the least aggressive with an overall 3.4% disease and 0.8 average DSR (Table 4). The average percent disease and raters'

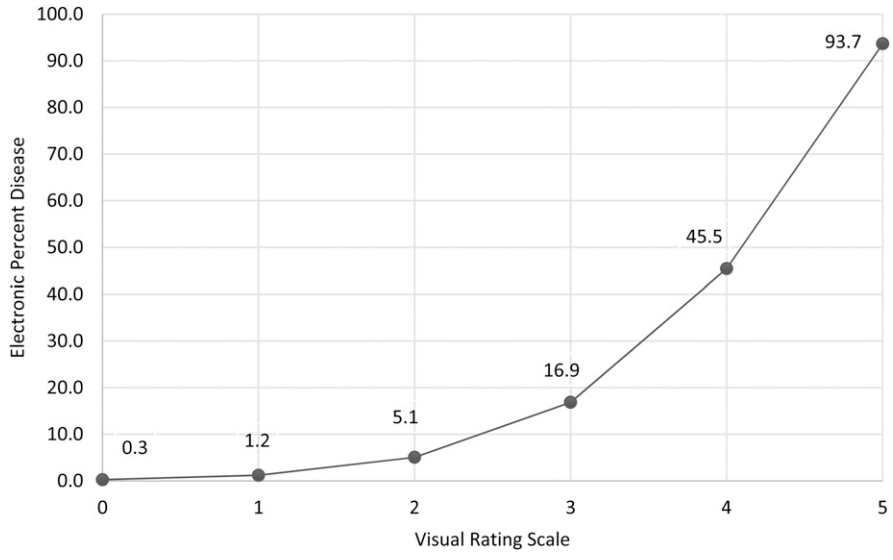

Fig. 2. Percent disease vs. the visual rater average disease severity rating (DSR) of detached strawberry leaves following inoculation with three isolates of *Colletotrichum* spp. Percent disease was calculated from computer image analysis of photographed leaves. Rater average DSRs are based on the average of two visual raters' DSRs.

Table 4. Percent disease from image analysis equivalent to visual disease severity rating (DSR) categories 0 to 5 of detached strawberry leaves following inoculation of 31 cultivars and 46 US and MSUS clones with three isolates of *Colletotrichum* spp.[z]

	Overall		Cultivar		US or MSUS	
Colletotrichum isolate	Disease (%)	Rater DSR	Disease (%)	Rater DSR	Disease (%)	Rater DSR
C. fragariae Cf63	3.4	0.8	6.9	1.3	1.7	0.6
C. fragariae Cf75	10.0	1.4	19.1	2.3	5.7	0.9
C. gloeosporioides Cg162	7.1	1.1	13.3	1.8	4.2	0.8

[z]Average percent disease and DSR from three trials with four replications each for each of the three *Colletotrichum* isolates for all 77 clones inoculated (overall) and for the cultivars and US and MSUS clones. (Data of control leaves are not included.)

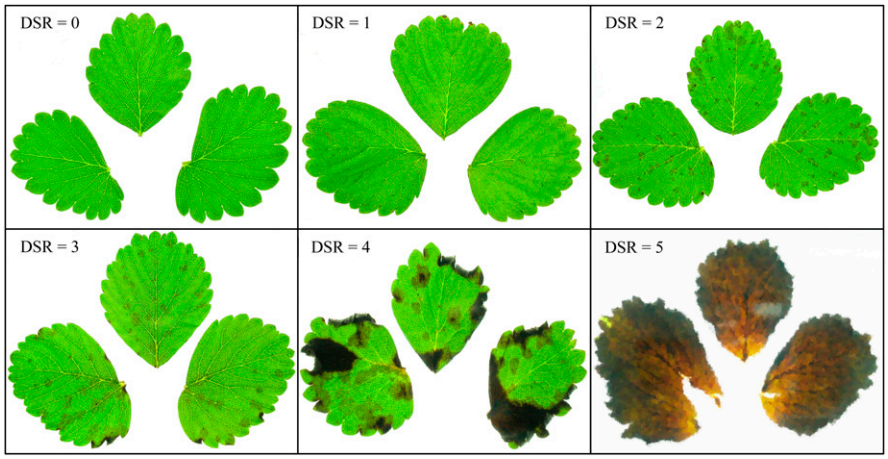

Fig. 3. Proposed 0 to 5 visual disease severity rating (DSR) scale for anthracnose disease caused by *Colletotrichum* spp. on detached strawberry leaves based on percent disease: DSR = 0 (0% to 0.7% disease); DSR = 1 (>0.7% to 3.5% disease); DSR = 2 (>3.5% to 10% disease); DSR = 3 (>10% to 35% disease); DSR = 4 (>35% to 70% disease); DSR = 5 (>70% disease).

average DSR were then separated by the source of the leaves (named cultivar or US and MSUS clone) because the US and MSUS clones were bred to be resistant to *C. fragariae*, and the isolates remained in the same order of aggressiveness. The difference between the ratings of the cultivars and the ratings of the MSUS clones (Table 4) indicates the MSUS clones are more resistant to all of the isolates. The percent disease of the two cultivars used for comparison were 'Chandler' for susceptible (Cf63 = 2.2%, Cf75 = 38.8%, and Cg162 = 16.7%) and 'Pelican' for resistant (Cf63 = 0.7%, Cf75 = 0.5%, and Cg162 = 2.4%). Across the three isolates, both the average DSR of the 31 cultivars (1.7) and the average percent disease (11.1%) were significantly higher than those of the 46 US and MSUS clones (DSR = 0.7 and percent disease = 5.5%) (data not shown). The overall average and the within fungal species average percent disease and DSR of each clone used in this study are given in Table 1. The DSRs of clones with a resistant response (DSR ≤ 0.8) are indicated in bold font and those with a susceptible response (DSR > 1.6) are indicated in italic font.

Discussion

Various disease rating methods have been used to assess disease severity and incidence, including percentage or ordinal rating scales, with or without reference photographs of diseased plant organs with associated ratings to help the visual rater in determining the correct rating. When choosing a disease rating method, the purpose of the research, as well as the time, practicality, cost, and accuracy of the ratings, must all be considered. Visual ratings can be used to assess disease severity quickly and may be used by farm advisors for rapid decisions on pesticide use in production fields. Electronic percent disease ratings made from image analysis give a more precise measure of lesion area than visual disease ratings and may be used when the research requires more detailed observations such as detecting and quantifying plant disease. Regardless of rating method, low (<10%) and high (>90%) levels of symptoms on plant tissues are easier to distinguish visually compared with symptoms that affect 10% to 90% of the plant tissue. In this study, the association between the two raters' DSRs was good (r_p = 0.87) with exact agreement on 52% of the total DSRs and varying amounts of agreement on the DSRs of the other 48%. Midrange ratings had the least amount of agreement. This variation may be explained by visual bias and is supported by previous research (Horsfall and Barratt, 1945; James, 1977; Sherwood et al., 1983; Slopek, 1989).

A rating scale with few categories (as the 0 to 5 scale in this study) presents the possibility of rater agreement by pure chance (Gwet, 2012). The weighted Kappa coefficient is a quantitative measure of the magnitude of the interrater agreement between the two raters' DSRs, and the value of k_w = 0.80 obtained in this study indicates a much better than chance agreement. The raters were in exact agreement on 52% of the total DSRs with 30% agreement at the lowest DSR of 0 and 16% agreement at the DSR of 1. The use of a 0 to 5 ordinal scale is sufficient to distinguish between susceptible and resistant strawberry leaves. An element of training, such as providing the visual raters with photographs representing each category on the rating scale, should improve the rating process and lead to a higher level of agreement between raters (James, 1977).

We propose a visual 0 to 5 disease rating scale as shown in Fig. 3 for classifying anthracnose disease caused by *Colletotrichum* spp. on detached strawberry leaves. This scale is based on percent disease determined by image analysis: DSR = 0 is equivalent to 0 to 0.7% disease, DSR = 1 is equivalent to >0.7 to 3.5% disease, DSR = 2 is equivalent to >3.5% to 10% disease, DSR = 3 is equivalent to >10% to 35% disease, DSR = 4 is equivalent to >35% to 70% disease, and DSR = 5 is equivalent to >70% disease. Prior research (Miller-Butler et al., 2018) indicated that detached leaf DSRs ≤0.8 indicate a resistant response based on a strong correlation between the detached leaf assay and the whole plant assay which has been used for more than 30 years to identify strawberry plants with resistance to anthracnose crown rot caused by *C. fragariae* and *C. gloeosporioides* (Galletta et al., 1993; Lewers et al., 2004, 2017; Smith et al., 1998). When rating leaves in the detached leaf assay, we suggest that a DSR of ≤0.8 based on the visual scale shown in Fig. 3 indicates a resistant plant. This photographic scale will be valuable to the visual rater and offers the novice an easy to use method that may also be used for assessment of other foliar diseases of strawberry.

Among the three isolates used in this study, *C. fragariae* isolate Cf75 was the most aggressive with an overall average percent disease of 10% and raters' average DSR of 1.4; *C. gloeosporioides* isolate Cg162 was the next most aggressive isolate; and *C. fragariae* isolate Cf63 was the least aggressive. Screening with an aggressive isolate helps to efficiently separate the more susceptible germplasm from the more resistant germplasm.

Detached leaf inoculations can be used as a preliminary screen to separate anthracnose-susceptible from resistant germplasm in large populations within breeding programs. The assessment of the germplasm (whether by image analysis or visual rating) can be fine-tuned higher or lower to separate susceptible from resistant germplasm by adjusting the scale to a more or less severe rating based on the breeders' objectives. Image analysis is more accurate and precise than visual disease ratings and was used as a "yardstick" in this study to compare percent lesion area obtained from the image analysis to the visual disease ratings. Comparison of visual assessments with image analysis of anthracnose disease on inoculated detached strawberry leaves was the main objective of this study. The visual 0 to 5 disease rating scale used was sufficient to separate susceptible from resistant strawberry leaves. The degree of agreement between the two methods (visual ratings and image analysis) used in this study was determined to be positively related (r_p = 0.79) and supports a preliminary study (Miller-Butler et al., 2013) that also found a strong correlation between image analysis and visual disease ratings.

Image analysis is expected to give a precise disease rating and is appealing as a means of assessing percent lesion area, but a disadvantage of image analysis is the considerable time required to prepare and photograph each leaf blade and to mark each lesion electronically. It can be difficult to distinguish between healthy and diseased tissue when visually rating leaves under laboratory lights, especially if the lesions are very small, but backlighting, photographing, and increasing picture magnification

of the lesions before analysis improved their visibility (Miller-Butler et al., 2013). Initial screening of large strawberry seedling populations for anthracnose resistance can be accomplished by field screening; however, if the lack of natural inoculum or the possible introduction of new strains of a pathogen prevents field screening, then preliminary screening using this detached leaf assay and visual disease assessment provides a reliable alternative. Seedlings identified as anthracnose resistant could then be established in field trials and evaluated for plant and fruit characteristics, as well as for resistance to other diseases and insects. Image analysis could be used for final decisions of anthracnose resistance or susceptibility before releasing a new cultivar. The choice of a disease rating method is dependent on the researcher's necessity for precision. This detached leaf assay is especially suitable for identifying anthracnose resistance in breeding programs with limited progeny or where greenhouse or field screening is not feasible for risk of introducing the pathogen into the area. In addition, it is ideally suited for assessing the resistance/susceptibility of parental breeding lines to various isolates and *Colletotrichum* species and mapping germplasm for resistance genes, and has been used in pesticide development studies (Abril et al., 2009).

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