Assessment of Severity of Powdery Mildew Infection of Sweet Cherry Leaves by Digital Image Analysis

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Abstract. A personal computer-based method was compared with standard visual assessment for quantifying colonization of sweet cherry (Prunus avium L.) leaves by powdery mildew (PM) caused by Podosphaera clandestina (Wallr.:Fr.) Lev. Leaf disks from 14 cultivars were rated for PM severity (percentage of leaf area colonized) by three methods: 1) visual assessment; 2) digital image analysis; and 3) digital image analysis after painting PM colonies on the leaf disk. The third technique, in which PM colonies on each leaf disk were observed using a dissecting microscope and subsequently covered with white enamel paint, provided a standard for comparison of the first two methods. A digital image file for each leaf disk was created using a digital flatbed scanner. Image analysis was performed with a commercially available software package, which did not adequately detect slight differences in color between PM and sweet cherry leaf tissue. Consequently, two replicated experiments revealed a low correlation between PM image analysis and painted PM image analysis ($r^2 = 0.66$ and $0.46$, $P \leq 0.0001$), whereas visual assessment was highly correlated with painted PM image analysis ($r^2 = 0.88$ and $0.95$, $P \leq 0.0001$). Rank orders of the 14 cultivars differed significantly ($P \leq 0.05$) when PM image analysis and painted PM image analysis were compared; however, rankings by visual assessment were not significantly different ($P > 0.05$) from those by painted PM image analysis. Thus, standard visual assessment is an accurate method for estimating disease severity in a leaf disk resistance assay for sweet cherry PM.

Powdery mildew (PM) of sweet cherry (Prunus avium L.) is commonly seen in Washington State orchards beginning in late April to mid-May (Grove and Boal, 1991a, 1991b). Although this disease is rarely present in most other major sweet cherry production areas, it is the most prominent preharvest disease in the arid fruit production regions of eastern Washington (Grove, 1991; 1998; Grove and Boal, 1991a, 1991b; Olmstead et al., 2000b). Major financial losses can occur from reductions in fruit quality (Grove, 1991). In addition, site-specific occurrences of $P. clandestina$ resistance to certain demethylation-inhibiting (DMI) fungicides, which are commonly used in cherry orchards, have been reported (Grove, 1997). In response to these issues, a breeding project to develop sweet cherry cultivars resistant to $P. clandestina$ was initiated at Washington State Univ.'s Irrigated Agricultural Research and Extension Center (WSU-IAREC) in 1998 (Olmstead et al., 2000a).

To reduce the time and resources required for identification of PM-resistant parents and subsequent screening of progeny, a screening assay was developed using leaf disks (Olmstead et al., 2000b). This assay optimized the conditions for $P. clandestina$ growth while maintaining leaf explants in a healthy condition. After 14 d in culture, leaf disks were rated for percentage of PM colonization by visual estimation, a common method used in field evaluations of sweet cherry PM (Gary Grove, pers. comm.).

Visual estimation is often the simplest and most rapid disease assessment method available; however, it is subjective and less precise than other disease assessment methods (Chung et al., 1997; Nutter et al., 1993). Rating scales, usually based on logarithmic progression from the median value of 50%, have been used in an attempt to alleviate the potential lack of accuracy and precision (Hebert, 1982; Horsfall and Barratt, 1945). Use of standard area diagrams (James, 1971), often incorporating logarithmic scales, is a common way to rate plants for disease severity. However, the use of such diagrams is subject to operator bias, and a different set of diagrams is required for each pathosystem (Lindow and Webb, 1983; Tucker and Chakraborty, 1997). Sherwood et al. (1983) reported that even when using standard area diagrams, both area and number of disease lesions could differ substantially from the actual area and number.

The limited precision and accuracy when visually assessing plant disease, even when coupled with tools such as standard area diagrams, has led to the development of computer-based systems for plant disease assessment (Kampmann and Hansen, 1994; Lindow and Webb, 1983; Martin and Rybizcki, 1998; Niemira et al., 1999; Tucker and Chakraborty, 1997). Advances in computer hardware and software have made powerful computing systems routinely available to most laboratories. To examine the hypothesis that digital image analysis may be more accurate and precise than visual assessments of colonization of cherry leaves by $P. clandestina$, a standardized leaf disk assay was used to compare resistance ratings from visual assessment vs. readily available commercial image analysis tools.

Materials and Methods

Sample preparation. Sweet cherry leaf disks (30-mm diameter) from 14 genotypes (‘Bing’, ‘Black Republican’, ‘Black Tartarian’, ‘Chelan’, ‘Lambert’, ‘Lapins’, ‘Moreau’, PMR-1, ‘Rainier’, ‘Sam’, ‘Stella’, ‘Tieton’, ‘Van’, and ‘Venus’) were collected, prepared, inoculated, incubated, and rated visually as described previously (Olmstead et al., 2000b). Using detached leaves collected in an orchard near Prosser, Wash., 30-mm-diameter leaf disks were cut with a cork borer and treated for 30 s in 70% aqueous ethanol. Leaf disks were then rinsed with sterile, distilled water and allowed to dry completely in a fume hood. The abaxial sides of the leaf disks were inoculated with an average of 25 conidia/mm², using a settling spore tower. Source leaves were uniformly heavily infected with $P. clandestina$. Leaf disks were inoculated by block according to the experimental design. Inoculated leaf disks were placed individually in sterile, plastic petri dishes (100 × 15 mm) on a single piece of filter paper saturated with a 1% sucrose solution. Petri dishes were randomized in a controlled environment growth chamber with white fluorescent lights (Precision Scientific Group, Chicago) and incubated at 22 ± 2 °C under a 14-h photoperiod [photosynthetic photon flux (PPF) = 50 μmol·m⁻²·s⁻¹]. The experimental design was a randomized complete block, with five replications per cultivar. The amount of leaf area covered by PM was estimated visually by an all-inclusive percentage of scale on the 14th day using a dissecting microscope (Bausch and Lomb, Rochester, N.Y.) at x30. The percentage of PM area was...
estimated visually and quantified using two methods of image analysis. The experiment was performed twice within 4 weeks.

**Scanning method.** Immediately after visual rating was completed for each sample, a digital image (Fig. 1A) was created by scanning the abaxial (inoculated) side of the leaf disk into a computer file using a standard flatbed scanner (Microtek ScanMaker E3; Microtek Lab, Redondo Beach, Calif.) at a resolution of 300 dots per inch (dpi) (118 dots per cm). To increase the efficiency of the scanning operation, each block (consisting of the 14 different cultivars described above) was digitized simultaneously. After scanning, individual leaf disks were cropped out of the larger picture, renamed according to cultivar and block number, and saved for later analysis as a .jpg file. The .jpg compressed file format was most amenable to the number and resolution of individual pictures to be analyzed.

**Image analysis.** Image files generated previously were opened using commercial software (SigmaScan Pro, ver. 4.0; Jandel Scientific Software, San Rafael, Calif.) for image analysis. To relate the pixel distance at 300 dpi to a known length, a standard image was generated by scanning a 30-mm long line drawn on paper. Using the software’s two-point calibration function, a standard pixel length for 30-mm at 300 dpi was measured. Distances in all sample image files were then calibrated with this information.

Although 30-mm-diameter leaf disks were used, edge imperfections and some marginal necrosis prevented quantification of total leaf disk area within a perfect circle. To define the appropriate total area for each leaf disk, the software’s trace measurement function was used to trace the circumference of each leaf disk image. The area within the trace line was then quantified using the measure area function.

Identification of PM area in the images was accomplished using the color threshold function. A sample of 10 random images was used to define and establish the correct color threshold for PM. The hue and saturation values for PM identified from these 10 images were used as the basis for identification of PM in the image files from the experiment. A red overlay was applied to illustrate the PM area on each picture according to the color threshold (Fig. 1B). The image analysis software consistently identified a “halo” of PM outside the leaf disks (Fig. 1B), which was attributed to an artifact of light reflection during the scanning operation. A correction factor of 3%, based on the average PM area assigned to the “halo” of resistant cultivars, was used for all image files. The software’s measure area function then quantified (in mm²) the total area of the red overlay. Percentage of PM area was calculated by dividing the PM area by total leaf disk area.

**Microscopic delineation of PM area.** To compare the precision of visual ratings and digital image analyses, a standard for PM area was determined for each leaf disk. After creating the scanned image .jpg files for each leaf disk, the PM area was examined with a dissecting microscope at ×30. Each individual PM colony or area was painted, using a very fine-tipped brush, with white enamel paint (Fig. 1C). The paint was allowed to dry for 1

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**Table 1.** Kruskal-Wallis analysis of variance for three methods of determining powdery mildew area on leaf disks of 14 sweet cherry cultivars in two experiments.

| Source of variation | Degrees of freedom | Sum of squares | Mean square | F value | Pr > F | Degrees of freedom | Sum of squares | Mean square | F value | Pr > F |
|---------------------|--------------------|----------------|-------------|---------|--------|--------------------|----------------|-------------|---------|--------|
| Painted area        |                    |                |             |         |        |                    |                |             |         |        |
| Block               | 4                  | 129.32         | 32.33       | 0.37    | 0.8324 | 4                  | 325.25         | 81.31       | 1.31    | 0.2784 |
| Cultivar            | 13                 | 23,842.4       | 1834.03     | 20.71   | 0.0001**| 13                 | 25,024.7      | 1,924.98    | 31.02   | 0.0001**|
| Error               | 52                 | 4,605.28       | 88.56       |         |        | 52                 | 3,227.05      | 62.06       |         |        |
| Visual assessment   |                    |                |             |         |        |                    |                |             |         |        |
| Block               | 4                  | 416.96         | 104.24      | 1.68    | 0.1683 | 4                  | 264.82         | 66.21       | 0.93    | 0.4556 |
| Cultivar            | 13                 | 23,969.4       | 1,843.8     | 29.74   | 0.0001**| 13                 | 23,689.9      | 1,822.3     | 25.51   | 0.0001**|
| Error               | 52                 | 3,223.64       | 61.99       |         |        | 52                 | 3,714.28      | 71.43       |         |        |
| Image analysis      |                    |                |             |         |        |                    |                |             |         |        |
| Block               | 4                  | 691.74         | 172.93      | 2.11    | 0.0933 | 3                  | 112.75         | 370.92      | 3.23    | 0.0325*|
| Cultivar            | 13                 | 22,465.55      | 1,728.12    | 21.07   | 0.0001**| 13                 | 9,041.88      | 695.53      | 6.06    | 0.0001**|
| Error               | 51                 | 4,183.66       | 82.03       |         |        | 39                 | 4,474.88      | 114.74      |         |        |

*All image files from one block were removed from analysis because of bulb failure during the scanning operation.
*Image files of the cultivar Van were not available for one block.
**Significant at $P \leq 0.05$ and 0.001, respectively.

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Fig. 1. Computer image files of a sweet cherry leaf disk before and after preparation for digital image analysis. (A) Leaf disk immediately after scanning operation. (B) Powdery mildew (PM) area (red) of leaf disk after digital image analysis. (C) Painted PM area of leaf disk. (D) Digital image analysis of painted PM area.
h, and the painted leaf disks were subsequently analyzed as described above (Fig. 1D). Use of the digital image analysis software in this manner was similar to use of the software planimeter described by Tucker and Chakraborty (1997).

Statistical analysis. Data from both leaf disk tests were analyzed using the Kruskal-Wallis nonparametric general linear model in SAS (SAS Institute, 1985). The Wilcoxon rank sum test ( Ott, 1993), a nonparametric procedure, was used to compare the ranking of the cultivars as measured by visual estimation or PM image analysis vs. painted PM image analysis. Assessments made by visual estimation and PM image analysis were regressed on those made by painted PM image analysis results.

Results

Four of the 14 cultivars used in the experiment were resistant to PM; thus, the assessment results did not adhere to a normal distribution. Therefore, all assessments were ranked, and nonparametric analyses were performed on those rankings. After pooling the data, results of the two experiments differed significantly (P ≤ 0.0001) for the digital image analysis ranking. Therefore, for each of the three analysis methods, the two experiments were analyzed separately (Table 1). A bulb failure in the scanner during the scanning operation in Expt. 2 caused a significant difference in light intensity for one replication. The digital image analysis for that replication was therefore ignored in the final analyses.

Accuracy and precision. Although the software’s user-defined thresholds for .jpg image file analysis appeared to represent the PM area on the leaf disks, digital assessments were much lower than either visual assessments or painted PM image analyses (Fig. 1, Table 2). Measurement of the painted PM image analysis was assumed to be the most precise measurement because it was determined by microscopic observation. Comparison of error sums of squares to total sums of squares (Chungu et al., 1997) was used to test this assumption. The error sums of squares (Table 1) for the painted PM image analysis represented 16.1% (Expt. 1) and 11.3% (Expt. 2) of the total error, compared with 11.6% and 13.4% for visual assessment, and 15.3% and 30.5% for PM image analysis. Visual assessment was the most precise in Expt. 1, painted PM image analysis in Expt. 2. Except for PM image analysis in Expt. 2, the magnitude of the difference in precision was much less than expected (Chungu et al., 1997; Nutter et al., 1993). A linear relationship \[ r^2 = 0.88 \text{ and } 0.95 \] (P ≤ 0.0001), respectively, in Expts. 1 and 2 was detected between cultivar ranks for visual assessment and painted PM image analysis (Figs. 2, 3). The correlation between PM image analysis and painted PM image analysis was much lower \[ r^2 = 0.66 \text{ and } 0.46 \] (P ≤ 0.0001), respectively. For PM image analysis, deviations from the regression line were greatest at low to intermediate infection levels (Figs. 2 and 3).

Comparison of rank order. Although the area measured by PM image analysis was obviously different from that of either visual assessment or painted PM image analysis, the ranked orders of the 14 cultivars were tested for similarity (based on the Wilcoxon rank sum test) between the three measurement methods (Table 2). The ranked orders of painted PM image analysis and visual assessment did not differ significantly (P > 0.05). However, cultivar rankings based on PM image assessment were significantly different from those based on painted PM image analysis (P ≤ 0.05).

Discussion

Digital image analysis systems have been used successfully to quantitatively assess disease area assessment in several pathosystems (e.g., Blanchette, 1982; Chungu et al., 1997; Lindlow and Webb, 1983; Martin et al., 1999; Niemira et al., 1999; Tucker and Chakraborty, 1997). However, with the exception of Niemira et al. (1999), these systems have required either specialized equipment or expertise in software application development to detect diseased areas. The objective of our experiments was to test a commercially available digital image analysis method that would not require specialized computer programming for implementation into a disease assessment program.

The digital image analysis method developed in these experiments measured cherry leaf PM area, but were not superior to standard visual assessment. Limitations in the adaptability of digital image analysis for disease

| Genotype          | Expt. 1       | Expt. 2       |
|-------------------|---------------|---------------|
|                  | Painted area  | Visual assessment | Image analysis | Painted area  | Visual assessment | Image analysis |
| Bing              | 66 ± 11       | 77 ± 15       | 20 ± 13       | 60 ± 19       | 58 ± 22       | 10 ± 11       |
| Black Republican  | 64 ± 13       | 77 ± 14       | 17 ± 14       | 46 ± 32       | 45 ± 37       | 6 ± 8        |
| Black Tartarian   | 5 ± 4         | 14 ± 7        | 1 ± 1         | 5 ± 3         | 9 ± 7         | 1 ± 1         |
| Chelan            | 0 ± 1         | 0 ± 0         | 0 ± 0         | 0 ± 0         | 0 ± 0         | 0 ± 0         |
| Lambert           | 0 ± 1         | 3 ± 2         | 0 ± 1         | 3 ± 2         | 5 ± 0         | 0 ± 1         |
| Lapins            | 17 ± 12       | 23 ± 16       | 4 ± 2         | 21 ± 17       | 22 ± 16       | 3 ± 6         |
| Moreau            | 0 ± 0         | 0 ± 0         | 0 ± 1         | 0 ± 0         | 0 ± 0         | 0 ± 1         |
| PMR-1             | 0 ± 0         | 0 ± 0         | 1 ± 1         | 0 ± 0         | 0 ± 0         | 0 ± 1         |
| Rainier           | 32 ± 18       | 45 ± 22       | 5 ± 3         | 29 ± 20       | 28 ± 19       | 1 ± 1         |
| Sam               | 26 ± 16       | 30 ± 26       | 8 ± 7         | 80 ± 6        | 84 ± 4        | 5 ± 4         |
| Stella            | 13 ± 3        | 23 ± 7        | 4 ± 1         | 14 ± 10       | 16 ± 10       | 0 ± 1         |
| Tieton            | 5 ± 3         | 15 ± 6        | 2 ± 1         | 27 ± 18       | 25 ± 14       | 2 ± 2         |
| Van               | 15 ± 13       | 19 ± 13       | 1 ± 1         | 31 ± 18       | 23 ± 12       | 0 ± 1         |
| Venus             | 0 ± 0         | 0 ± 0         | 0 ± 1         | 0 ± 0         | 0 ± 0         | 0 ± 1         |

Ranking based on Wilcoxon rank sum test, with 1 = least susceptible, 14 = most susceptible.
assessment have been reported before. Chung et al. (1997) found digital image analysis to be unable to distinguish between corn (Zea mays L.) kernels with symptoms of Fusarium graminearum Schwabe and those without. When the diseased area did not differ sufficiently in color from healthy plant material, Blanchette (1982) was unable to distinguish the diseased area by digital image analysis without staining. Powdery mildew area on cherry leaf disks appeared to be identified reliably by user-defined color thresholds in the present experiments; however, quantification of PM area by digital image analysis was less accurate than visual or painted PM image assessment. Differences in color and intensity between healthy and colonized areas of the leaf disk may not have been great enough for precise measurement, as suggested by the fact that the digital image analysis software was able to accurately quantify PM area painted a uniform white. A method of staining the PM area prior to digital image analysis might improve the contrast between diseased and healthy tissue. Alternatively, scanning at a resolution higher than 300 dpi might facilitate determination of the difference between healthy and colonized areas. Although the technique used in this study was not suited for sweet cherry PM, a pathosystem with denser sporulation may provide the contrast needed between healthy and colonized plant tissue to accurately quantify PM area.

Rather than determining the size of infected areas, measuring the components of resistance, a tactic used successfully by Kampmann and Hansen (1994) for digital image analysis of PM on cucumber (Cucumis sativus L.) may be feasible. For example, P. clandestina overwinters by formation of cleistothecia. Numerous brown-to-black cleistothecia can be seen on the most susceptible cultivars after 14 d in the leaf disk assay. Because the color contrast between cleistothecia and healthy tissue is more distinct than that between whole colonies and healthy tissue, counting numbers of cleistothecia as a measure of disease susceptibility may be possible, provided that two mating types of P. clandestina are present in the original inoculum and cleistothecial formation is not affected by host plant resistance.

In each of the three assessment methods, subjective decisions made by the assessor were involved. Visual assessment was the most subjective, requiring the identification of diseased area and estimation of the percentage of colonization. The digital image analysis software required the development of user-defined color thresholds that identified PM, and all subsequent measurement of colonized area was automated. Although painted PM area was used as a standard in these experiments, there are potential sources of error associated with this technique. To visualize discrete PM colonies, a dissecting microscope was required; however, the area to be painted was still identified subjectively. The area visually interpreted as colonized by PM may not actually be composed of contiguous PM colonies. Under microscopic evaluation, one can see that there are areas surrounding the conidiophores of P. clandestina that are not actually colonized. A fine-tipped paintbrush was used, but accurately reproducing each PM conidiophore on the leaf was not possible, resulting in potential over-estimation of diseased area. Conversely, digital image analysis was not able to identify precise areas of PM colonization that could be seen clearly under a dissecting microscope, resulting in potential underestimation of diseased area. Although measurement of the painted PM area may not correspond to actual PM colonization, it was less subjective than was strict visual assessment. The utility of the digital image analysis system was reduced by the inability to delineate cultivars with low levels of PM colonization, a function that is necessary in breeding for disease resistance.

The use of plant material from a detached leaf disk test made image capture relatively simple. By using a flatbed scanner, much of the illumination problems encountered by other authors (Lindow, 1983; Lindow and Webb, 1983; Martin and Rybicki, 1998) were avoided. Although the software used in this experiment was able to measure grayscale images, color images were used because our preliminary
testing showed that leaf veins were often grouped in the same pixel intensity as diseased area when grayscale images were analyzed, a result similar to that of Martin and Rybicki (1998). The digital image analysis software used in this experiment has been used successfully to determine the susceptibility of cut potato (Solanum tuberosum L.) tubers to late blight [Phytophthora infestans (Mont.) de Bary] (Niemira et al., 1999). Measurement of the diseased area of tubers utilized the software’s intensity measurement function, a method that was not adequate for measurement of PM colonization in our experiments.

Although visual assessments often have been criticized, the additional time and resources involved in developing accurate digital image analysis systems for measurement of sweet cherry PM seem unnecessary, given the current limitations of commercially-available technology. In these experiments, a strong relationship was detected between painted PM image analysis and results from standard visual assessments. The extent of this relationship supports the utility of standard visual assessment for rating sweet cherry genotypes in the WSU-IAREC breeding program for PM resistance. However, comparison of visual assessments of diseased leaf tissue with subsequent painted PM image analysis may be a useful tool for training new personnel. In breeding programs that involve multiple or changing personnel in PM assessments, the objectivity of digitized PM colony quantification as described herein eventually may be useful, particularly if greater contrast or resolution of the scanned images can be achieved.

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