Effect of Detergent on the Quantification of Grapevine Downy Mildew Sporangia from Leaf Discs

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Abstract. Grapevine downy mildew (DM), caused by the oomycete *Plasmopara viticola* (Berk. & Curt.) Berlese & de Toni, is a major disease, especially in humid viticultural areas. Development of resistant cultivars is an important objective for grapevine breeding. To establish a reliable and inexpensive quantitative method to aid in breeding for DM resistance, we improved the method for counting the number of sporangia on leaf discs, and developed a method for counting the number of sporangia in solution. To prevent the loss of DM sporangia from adhesion onto plastic ware, we found as little as 0.01% Tween 20 was effective. On the other hand, this detergent was shown to have a severe inhibitory effect upon DM infection of leaves. We developed a sporangia counting method using dried droplets of DM suspensions, and the method was highly correlated with counting by hemacytometer ($R^2 > 0.96$). The nonparametric Spearman's rank correlations between visual rating and the number of the sporangia were as high as $\rho = 0.82$ to 0.91 , suggesting that evaluation by the visual rating could provide a good estimate of the sporangia numbers on leaf discs. We established a high-throughput and inexpensive method with acceptable accuracy for DM resistance evaluation based on a leaf disc assay, and our results suggested that visual ratings of infected leaf discs provide a good estimate of sporangia numbers.

Grapevine downy mildew occurs throughout the world, and is one of the most destructive diseases in viticultural areas with high annual rainfall. DM attacks all green parts of the vine, such as leaves, tendrils, shoots, inflorescences, and bunches. The lesions become covered with white, downy sporangio-phores when the weather is wet (Lafon and Bulit, 1981). The formation of sporangio-phores is very closely linked to the relative humidity, which must be at 95% to 100%

(Lafon and Bulit, 1981). Under these conditions the sporangiophores form within a day, but a minimum of 4-h darkness is necessary for sporangiophore formation (Lafon and Clerjeau, 1998). Individual foliar lesions sporulate repeatedly, but their productivity (sporangia/lesion) declines rapidly through repeated cycles of sporulation (Kennelly et al., 2007). Kortekamp and Zyprian (1999) mentioned that sporangia of *P. viticola* stick tightly onto wet and dry surfaces.

Resistance of *Vitis* germplasm to DM has been evaluated via either natural infection (Boso et al., 2014; Brown et al., 1999; Cadle-Davidson, 2008; Demaree et al., 1937; Pavloušek, 2012; Prajongjai et al., 2014; Wan et al., 2007), or artificial inoculation (Boso et al., 2014; Cadle-Davidson, 2008; Eibach et al., 2007; Prajongjai et al., 2014; Staudt and Kassemeyer, 1995). Fourteen downy mildew resistance loci (*Rpv1* to *14*) have been identified, and 20 molecular markers associated with 12 of these loci are available for use (Julius Kühn-Institut, 2014). Resistance of individuals was evaluated by

a leaf disc assay (Bellin et al., 2009; Blasi et al., 2011; Feechan et al., 2013; Fischer et al., 2004; Marguerit et al., 2009; Schwander et al., 2012; Venuti et al., 2013) or by observation of symptoms in the field (Bellin et al., 2009; Cadle-Davidson et al., 2011; Fischer et al., 2004; Moreira et al., 2009; Venuti et al., 2013). Some studies quantified sporangia numbers using a cell counter (Bellin et al., 2009; Blasi et al., 2011; Marguerit et al., 2009).

Field evaluation is a directly relevant method for rating DM resistance because breeders would like to be sure resistance is useful under field conditions. However, preparing a no-fungicide vineyard for all breeding populations is not always possible. And, since weather is variable, disease pressure could be too high or low. As an alternative method, we could evaluate resistance in controlled conditions using artificial inoculation, such as via a leaf disc assay (Bellin et al., 2009; Blasi et al., 2011; Feechan et al., 2013; Fischer et al., 2004; Marguerit et al., 2009; Schwander et al., 2012; Venuti et al., 2013) or a detached leaf assay (Kono et al., 2013).

Leaf disc assays are the standard method for controlled evaluation of DM resistance, and the number of DM sporangia produced were counted with the aid of either a hemacytometer (Bellin et al., 2009; Kennelly et al., 2007) or a cell counter (Bellin et al., 2009; Blasi et al., 2011; Marguerit et al., 2009). However, it is laborious to measure the concentration of many samples by a glass hemacytometer. Use of a cell counter is a high throughput alternative method, yet the equipment is expensive. Contamination of small particles could cause overestimation of the suspension concentration, and clumps of sporangia could cause underestimation.

In this study, we tried to establish a high-throughput and inexpensive method with acceptable accuracy for DM resistance evaluation based on the leaf disc assay. Because the sticky nature of grapevine DM sporangia to plastic ware or other vessels are not well described, we investigated the effect of detergent upon the adhesion between sporangia and plastic ware, as well as the effect upon DM infection. We also investigated whether the visual evaluation of sporangia density on leaf discs would provide a good estimate of sporangia numbers as determined by hemacytometer counts (with detergent) using three different breeding populations.

Materials and Methods

DM maintenance. A mixture of DM sporangia was collected from 'Riesling' leaves in Geneva, NY, on 7 Sept. 2010, and maintained as bulk mixtures on 'Chardonnay' and 'Riesling' leaves. Expanding leaves (no longer translucent and shiny) were harvested from either a greenhouse without fungicides or one maintained with sulfur applications, and surface sterilized for 2 min with 0.6% sodium hypochlorite, then washed with tap water and deionized water three times each. Washed leaves were placed on 1% (w/v) agar in 100 mm × 15 mm polystyrene petri

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plates (Fisher Scientific, Pittsburgh, PA). Sporangia suspensions in water ($\approx 2 \times 10^4$ sporangia/mL) were prepared and drop-inoculated onto the abaxial side of the leaves. Plates were incubated inside a plastic bag at 21 to 28 °C, (24 °C on average) with a 14-h light/10-h dark photoperiod. The next day, dishes were taken out of the bags and excess water on the inoculated leaves was wiped away with Kimwipes (Kimberly-Clark, Roswell, GA). Then, the dishes were sealed completely by parafilm (Pechiney Plastic Packaging, Menasha, WI) to maintain humidity, and were incubated in the same incubation room for several days. After sporangia were visible at 4 to 6 d postinoculation (dpi), they were inoculated onto new detached leaves as above for maintenance and propagation.

Effect of Tween 20 on the recovery of sporangia from plastic ware. Sporangia suspension in water (300 μ L of $\approx 1.5 \times 10^5$ sporangia/mL) was subsampled into 5 sets of 12 microcentrifuge tubes (60 tubes in total), and 3 μ L of Tween 20 solution (100 $\times$ of final concentration) or water was added to each set of tubes for final concentrations of $\approx 0\%$, 0.01%, 0.025%, 0.05%, and 0.1% Tween 20 (v/v). After 5 min of incubation without shaking, suspensions were shaken by hand, and 3 μ L of suspension was sampled to count sporangia by the dried droplet method as described below. Estimated log₁₀-transformed mean concentrations of each Tween 20 concentration were separated by Tukey's all-pair comparisons. R (R Core Team, 2013) was used for all statistical analysis in this study.

Effect of Tween 20 on DM infection. In 2012, sporangia suspensions (2×10^5 sporangia/mL) containing 0%, 0.01%, and 0.1% (v/v) of Tween 20 were inoculated onto detached greenhouse grown expanding 'Chardonnay' leaves (past the translucent and shiny stage). Inoculated leaves were incubated for 6 d and the symptoms were observed. In 2013, 14 leaves that were almost the same age as 'Chardonnay' leaves were harvested from 'Rizamat' and nine leaf discs from each leaf were placed on 1% agar medium in a 9-cm disposable petri dish. Sporangia suspensions (50 μ L of 5×10^5 sporangia/mL) containing the 0%, 0.01%, and 0.1% Tween 20 were inoculated onto three leaf discs in each dish, respectively. Inoculated discs were incubated in the same way as described in the DM maintenance section except as follows: 12 h of light (at 22 °C), and 12 h of dark (at 18 °C). At 6 dpi, each leaf disc was harvested into a 1.5-mL tube and stored at -20 °C until counting. We regarded each leaf as a replicate (14 replicates in total), and the mean sporangia number was calculated by the same method as described below for each Tween 20 concentration.

DM sporangia counting. The number of DM sporangia was counted using either hemacytometers or a "dried droplet" method. We used both a Bright-Line hemacytometer (Warner-Lambert Technologies, Buffalo, NY), and a disposable hemacytometer (INCITO C-Chip Disposable Hemacytometers,

INCITO Co., Chonan-si, Chungnam-do, Republic of Korea). Both hemacytometers were improved Neubauer types. Sporangia were also counted within a dried droplet of suspension (dried droplet method). Three microliters of suspension containing 0.01% (v/v) Tween 20 (H5152, Promega, Madison, WI) was dropped onto a slide and was dried completely on a dry heat block at ≈ 70 °C. After drying, droplets were mounted in a 0.05% Aniline Blue solution in 67 mM K₂HPO₄ (Sigma-Aldrich, St. Louis, MO) under cover slips. The total number of sporangia within a droplet was counted under a microscope, and the original suspension concentration was estimated.

The relationship between suspension concentration estimates by the dried droplet method and a hemacytometer. A highly concentrated sporangia suspension was obtained from diseased leaves, and the concentration was calculated by a hemacytometer. A dilution series of sporangia suspensions (1, 2, 4, 6, 8, 10, 20, 30, 40, 50 $\times 10^4$ sporangia/mL) containing 0.01% (v/v) Tween 20 was prepared in a separate tube for each concentration, and their concentration was then estimated by both the hemacytometer and the dried droplet method. Sporangia numbers were counted by a hemacytometer four times, and four dried droplets from each tube were also prepared for counting. Means of these four repetitions were log₁₀-transformed and subjected to regression analysis. The experiment was done twice.

Preparing sample leaf discs of grapevine breeding populations. We evaluated DM resistance of the three breeding populations; 'Horizon' $\times$ *V. cinerea* B9 (population HC, 91 individuals), 'Horizon' $\times$ Illinois 547-1 (*V. cinerea* B9 $\times$ *V. rupestris* B38; population HI, 91 individuals) and *V. rupestris* B38 $\times$ 'Horizon' (population RH, 91 individuals) in 2012. Populations HC, HI, and RH were planted on 26 May 2011, 1 and 2 June 1998, and 3 May 2010, respectively. For HC and RH, a mixture of boscalid (Endura 70WG) and captan (Captan 80WPG) were sprayed on 24 May and a mixture of metrafenone (Vivando 2.5SC), captan (Captan 80WPG), and phosphorus acid (ProPhyt) was sprayed on 8 June. For HI, mandipropamid (Revus 2SC) was sprayed on 24 May and phosphorus acid (ProPhyt) was sprayed on 8 June. Expanding leaves (no longer translucent and shiny) were sampled from different shoots for DM evaluation.

For population HC, four leaves were sampled on June 4. Harvested leaf sections were washed using a bleach solution as described above in "DM maintenance." Two leaf discs (1.5 cm diameter) were punched from each leaf with a cork borer, and were placed on 1% agar in a 100 mm $\times$ 15 mm polystyrene petri dish. For population HI, eight different leaves were sampled on 20 June and leaf discs were prepared in the same way as for population HC. For population RH, four leaves were sampled on 25 June. Two leaf discs (1 cm in diameter) were punched from each leaf with a cork borer, and were placed on 1% agar in a glass cake pan (25 cm $\times$ 33 cm, 4 L). Each

pan contained two discs from each leaf across all genotypes (91 individuals) and parents (*V. rupestris* B38, 'Horizon') and 'Chardonnay'. A randomized complete block design was used, with one pan for each of the four replicates. Pans were sealed with plastic wrap and then placed inside plastic bags to maintain humidity.

Artificial inoculation and evaluation for leaf discs from vineyards. A sporangia suspension in water (50 μ L of $\approx 5 \times 10^4$ sporangia/mL) was inoculated on each 1.5 cm leaf disc (population HC and HI), and 25 μ L of sporangia suspension in water was inoculated on each 1-cm leaf disc (population RH). Inoculated discs were incubated in the same way as described in the DM maintenance section. At 6 dpi, symptoms of each disc were visually rated, with a similar rating scale (Fig. 1) to the visual scale in Blasi et al. (2011). Description for the rating scores are as follows: 1) no sporangia, 2) very few sparsely distributed sporangia, 3) a few sporangia only seen in apparently smaller area than total inoculated site, 4) many sporangia but not confluent, and 5) almost confluent sporangia. The score was inverted from Blasi et al. (2011), but in the same order as Fischer et al. (2004), in accordance with the ascending order of counted sporangia number. After the visual rating, the number of sporangia was counted by the dried droplet method as described above (population HC and HI), or by use of a disposable hemacytometer to quantify a 100 μ L sporangia suspension in 0.01% (v/v) Tween 20 (population RH).

Analysis of correlation. The nonparametric Spearman's rank correlation coefficient (ρ) was calculated between mean sporangia number and mean rating scores by the statistical package R to analyze the correlation between mean rating scores and sporangia numbers for each individual. Then, to show the quantitative relationship between the two, regression analysis was done as follows. Since the variance of the sporangia number increased along with the mean, a logarithmic transformation (base 10) was applied to the data to make the variance independent of the mean. To avoid a zero count for the logarithmic transformation, Yamamura (1999) suggested adding half of a discrete unit before the logarithmic transformation. And therefore, we added half of the detection limit (i.e., discrete unit) of the sporangia number by the dried droplet method (25 and 50 per disc for populations HC and HI, respectively) or by hemacytometer (28 per disc for population RH) before transformation. Regression coefficients and intercepts were calculate with log-transformed sporangia number as the response variable, and mean rating scores as an explanatory variable using R.

Results and Discussion

Effect of Tween 20 on the recovery of sporangia from plastic ware. Sporangia of grapevine DM stick to plastic ware such as disposable petri dishes and microcentrifuge tubes (Fig. 2). This adhesion results in the loss

of sporangia from the suspension, potentially causing significantly reduced estimation of sporangia concentration. To prevent sporangia from attaching to plastic ware, we added a detergent, Tween 20, to the suspension and evaluated the effect. The difference of the suspension concentration between 0% Tween 20 and all other concentrations was almost 4-fold (Fig. 3). Hence, as little as 0.01% (v/v) Tween 20 effectively prevented sporangia from attaching to plastic ware. Without Tween 20, the suspension concentration would have been significantly underestimated owing to the sporangia loss on plastic ware.

Effect of Tween 20 on sporangia inoculation. In contrast, we found that even 0.01% Tween 20 almost completely inhibited DM infection on grapevine leaves (Fig. 4; Table 1). Hence, sporangia suspensions containing Tween 20 cannot be used for inoculation. In accordance with our finding, Stanghellini and Tomlinson (1987) showed that zoospores of *Pythium* and *Phytophthora* species ceased motility and lysed after being placed in solutions with the surfactant, Agral. Since DM sporangia release zoospores that swim to stomata to initiate infections, detergent may cause damage to zoospores before infection. Kortekamp and Zyprian (1999) applied Tween 20 to leaves of *V. davidii*, *V. cinerea* and *V. doaniana*, and observed successful infection of DM. However, they used 0.05% Tween 20 for 15 min and then washed leaves three times with sterile water, so there was no remaining Tween 20 on these leaves at the time of inoculation. This condition is crucially different from our inoculation with the same detergent, and could be the reason for successful infection in their report.

Measuring sporangia concentration by the dried droplet method. Sporangia concentrations are usually measured with a hemacytometer (Bellin et al., 2009; Kono et al., 2009) or cell counter (Bellin et al., 2009; Blasi et al., 2011; Marguerit et al., 2009). As a reliable and inexpensive alternative, we investigated the dried droplet method to estimate the concentration of sporangia in suspension (see Materials and Methods). To investigate the accuracy of the dried droplet method, we estimated the concentration of sporangia in a dilution series both by the dried droplet method and hemacytometer. As shown in Figure 5, the concentration of sporangia measured by a hemacytometer can be regressed with a high degree of accuracy with the estimate obtained from the dried droplet method. The slope was nearly one and highly significant ($P < 0.001$), whereas the y -intercept was not significantly different from zero ($P = 0.37$ and 0.78 for the first and second experiments, respectively). This means that both regression equations were considered to be no different from $y = x$. Hence, we concluded that the dried droplet method could be used to measure the concentration of sporangia suspensions containing 0.01% Tween 20 as accurately as a hemacytometer.

Fig. 1. Rating scale for visual rating of leaf discs. Range of the scale was set to be similar to the visual scale in Blasi et al. (2011) but modified to increase with increasing sporangia number. (1) No sporangia, (2) very few sporangia just seen sparsely, (3) a few sporangia only seen in apparently smaller area than total inoculated site, (4) many sporangia but not confluent, (5) almost confluent sporangia.

Fig. 2. Sporangia attached to plastic ware. (A) A sporangia suspension was inoculated onto plastic ware. (B: arrows) After removing the suspension, sporangia remained on the surface and can be seen as a whitish smear. Bars = 1 cm.

Fig. 3. Actual sporangia concentration in microcentrifuge tubes with 0.0% to 0.1% Tween 20. Mean separation by Tukey's honestly significant difference at $P \leq 0.001$.

The lower limit of the Improved Neubauer hemacytometer is one sporangia per whole chamber (0.9 mm^3), which is 1.1×10^3 sporangia/mL. Thus, from a statistical point of view, only suspensions more concentrated

than 1.1×10^3 sporangia/mL can be accurately quantified by hemacytometer. In contrast, by increasing the volume of a drop on a slide, the lowest measurable concentration from the dried droplet method could be

Fig. 4. Sporangia formed on a 'Chardonnay' leaf 6 d after inoculation with downy mildew sporangia at the indicated concentration of Tween 20.

Table 1. Effect of Tween 20 on success of downy mildew infection on 'Rizamat' leaf discs. Suspensions of 5×10^5 sporangia/mL in 0.0%, 0.01%, or 0.1% Tween 20 were inoculated and the resultant sporangia numbers on leaf discs were counted at 6 d postinoculation.

Tween 20 concn	Mean sporangia number/leaf disc ^z
0	2.1×10^4 a
0.01	27 b
0.1	2.9 b

^zMean separation in columns by Tukey's honestly significant difference at $P \leq 0.001$.

lower. For example, the lower limit of a 3 μ l dried droplet that we use throughout this study is 3.3×10^2 sporangia/mL, which is 3-fold lower than the Improved Neubauer hemacytometer. Moreover, by using a 10 μ l droplet, the limit could be 1×10^2 sporangia/mL. This flexibility and improved sensitivity are advantages of the dried droplet method, when working with less concentrated suspensions.

Correlation between rating score and sporangia number on leaf discs. Visual ratings can be labor-saving and high throughput when many samples must be evaluated, but could be less accurate or more subjective than sporangia counts. To evaluate the accuracy of visual ratings, we analyzed the relationship between mean visual rating scores and the mean sporangia number, counted by the dried droplet method on leaf discs, using three breeding populations: Horizon $\times$ *V. cinerea* B9 (population HC); Horizon $\times$ Illinois 547-1 (population HI); and *V. rupestris* B38 $\times$ Horizon (population RH), including the parents (Fig. 6). The Spearman's rank correlation coefficients between sporangia number and the log-transformed mean rating score within each population were as high as $\rho = 0.91$ (population HC; $P < 0.001$), $\rho = 0.89$ (population HI; $P < 0.001$), and $\rho = 0.82$ (population RH; $P < 0.001$), respectively. The linear regression equations for populations HC, HI, and RH with log-transformed sporangia number and mean rating score were $y = 0.58x + 2.0$ ($R^2 = 0.80$), $y = 0.54x + 2.5$ ($R^2 = 0.77$), and $y = 0.52x + 2.2$ ($R^2 = 0.70$),

respectively (Fig. 6). In other words, evaluation by the mean rating score provided a good estimation of sporangia numbers on leaf discs. The reason for the good correlation might be that the definition of our rating score is on the basis of the quantitative amount of sporangia, but not based on qualitative symptoms, such as the presence of hypersensitive response.

Many previous quantitative trait locus (QTL) analyses were done using evaluation data obtained by visual ratings, and many QTLs for DM resistance were discovered (Bellin et al., 2009; Blasi et al., 2011; Feechan et al., 2013; Fischer et al., 2004; Marguerit et al., 2009; Schwander et al., 2012; Venuti et al., 2013). Our results suggest that these QTLs could be interpreted as the QTLs for the number of sporangia after DM inoculation. To support this interpretation, some QTL analyses in previous studies were performed both by visual rating and counting the number of sporangia, and resulted in the

finding of the same QTLs (Bellin et al., 2009; Blasi et al., 2011; Marguerit et al., 2009).

In this study, we found that a low concentration of detergent helps with the handling of DM sporangia, yet it also effectively prevents DM infection on grapevine leaves. Counting the number of sporangia by the dried droplet method with detergent was as accurate as the counting with a hemacytometer. We also validated that the log-transformed number of sporangia on a leaf disc was strongly correlated with visual ratings. This latter method could be used as an accurate, inexpensive and high-throughput technique for the evaluation of many samples coming from biparental populations for QTL analyses, germplasm screening, parental material screening, and testing effects of DM control substances in a leaf disc assay system. Our results could also provide evidence that visual ratings of leaf discs reflect the number of sporangia on the disc.

Fig. 5. Relationship between the suspension concentration estimated by the dried droplet method and an improved Neubauer hemacytometer. The experiment was performed twice, and the results of the first (A) and the second (B) are shown. Nonsignificant (NS) or significant (***) at $P \leq 0.001$.

Fig. 6. Regression of log-transformed sporangia number as a function of mean rating scores on leaf discs from seedling vines in three hybrid populations. All leaf discs of each individual, including parents, were evaluated by both visual rating and by counting the sporangia number, and means were calculated for each individual. Closed circle: 'Horizon' $\times$ *V. cinerea* B9 (population HC). Open circle: 'Horizon' $\times$ Illinois 547-1 (*V. cinerea* B9 $\times$ *V. rupestris* B38; population HI). Cross: *V. rupestris* B38 $\times$ 'Horizon' (population RH). Solid line is the regression line for population HC, dashed line is for population HI, and dotted line is for population RH.

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