Simultaneous quantification of sporangia and zoospores in a biotrophic oomycete with an automatic particle analyzer: Disentangling dispersal and infection potentials

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Quantitative pathogenicity traits drive the fitness and dynamics of pathogens in agricultural ecosystems and are key determinants of the correct management of crop production over time. However, traits relating to infection potential (i.e. zoospore production) have been less thoroughly investigated in oomycetes than traits relating to dispersal (i.e. sporangium production). We simultaneously quantified sporangium and zoospore production in a biotrophic oomycete, for the joint assessment of life-cycle traits relating to dispersal and infection potentials. We used an automatic particle analyzer to count and size the sporangia and/or zoospores produced at t = 0 min (no zoospore release) and t = 100 min (zoospore release) in 43 Plasmopara viticola isolates growing on the susceptible Vitis vinifera cv. Cabernet Sauvignon. We were able to differentiate and quantify three types of propagules from different stages of the pathogen life cycle: full sporangia, empty sporangia and zoospores. The method was validated by comparing the sporangium and zoospore counts obtained with an automatic particle analyzer and under a stereomicroscope (manual counting). Each isolate produced a mean of 5.8 ± 1.9 (SD) zoospores per sporangium. Significant relationships were found between sporangium production and sporangium size (negative) and between sporangium size and the number of zoospores produced per sporangium (positive). However, there was a significant positive correlation between total sporangium production and total zoospore production. This procedure can provide a valid quantification of the production of both sporangia and zoospores by oomycetes in large numbers of samples, facilitating joint estimation of the dispersal and infection potentials of plant pathogens in various agro-ecological contexts.

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1. Introduction

Crop pathogens have to adapt to a broad range of selective pressures, such as changes in cropping practices, new host plants and climate change (Anderson et al., 2004; Burdon and Thrall, 2008). The adaptation of pathogens to local agro-ecological conditions depends on the evolution of quantitative pathogenicity traits as for example latency period, lesion size, infection efficiency and spore production rate which are major components of pathogen aggressiveness (Pariaud et al., 2009; Lannou, 2012). Studying the variability of pathogenicity traits might therefore prove a useful proxy for assessing pathogen adaptive potential that determines disease epidemic development in agricultural ecosystems.

The most commonly measured quantitative traits of fungal plant pathogens concern the asexual cycle of infection. Spore production rate is the trait most widely used by plant pathologists (Pariaud et al., 2009; Lannou, 2012), because it is a strong determinant of aggressiveness and transmission capacity (i.e. the capacity of the pathogen to disperse and infect new hosts). It corresponds to the number of spores produced per lesion and per unit time (Alizon et al., 2009; Pariaud et al., 2009). In oomycetes, estimates of spore production are based on sporangium production rates, which have been shown to vary with host genotype (Delmottet al., 2014; Rouxel et al., 2013), ecological environment (Davidson et al., 2011), exposure to fungicides (Stein and Kirk, 2003) and host physiological status (Cohen and Rotem, 1970). However, in most of oomycetes, the sporangia are not the propagules actually responsible for infection success (exceptions include Peronospora sp. and some Phytophthora sp. in which sporangium undergoes direct germination). This role is fulfilled by zoospores the wall-less, biflagellate, uninucleate cells present in the sporangia (reviewed in Walker and West, 2007). Oomycete life cycles have sexual and asexual phases (Erwin and Ribeiro, 1996; Gessler et al., 2011). The sexual oospores mature over the winter, germinate and form sporangia following exposure to a water film. The asexual cycle involves the production of infectious...
spores (sporangia containing swimming zoospores), dispersal and the reinfection of host tissue. These stages require the combination of a suitable temperature and moisture. The survival of zoospores, the principal infection unit of oomycete pathogens, determines infection success (Diéguez-Uribondo et al., 1994; Pistinini et al., 2014).

In experimental studies of pathogenic oomycetes (mostly performed on Phytophthora sp.), the production (Eye et al., 1978; Wilkinson et al., 2001; Dalio et al., 2014), mobility (Pezet et al., 2004), viability (Galiana et al., 2005; Ahonsi et al., 2010) and germination rate (Matheron and Porchas, 2000; Galiana et al., 2005) of zoospores have been studied. Zoospore production has been reported as a tool to standardize inoculum suspensions before infection experiments (Stein and Kirk, 2003) and to evaluate the fitness of isolates (Porter et al., 2007). Both indirect and direct methods can be used to monitor zoospore production in oomycetes. For example, the number of zoospores per leaf has been estimated indirectly from colony numbers (Davidson et al., 2005; Davidson et al., 2011). The direct counting of zoospores may be achieved by the manual counting of swimming zoospores in microdrops, placing on agar, the counting of immobilized zoospores in a hemocytometer under a stereomicroscope and the use of an electronic particle counter, as first proposed by Schoulities and Yang (1970). Manual zoospore counting is the most widely used method. The principal advantage of this method is its ability to distinguish between motile and immobilized zoospores (Harris, 1986), but the number of samples that can be processed and the number of zoospores that can be counted are limited. A method for the automatic quantification of sporangia and zoospores in a large number of pathogen isolates is required to improve the accuracy of quantitative trait description at the individual and population scales (via a high-throughput of samples and traits analyzed). Automatic zoospore quantification with a particle analyzer requires zoospore fixation, but this method can be used to monitor the large number of samples required in populational and microevolutionary studies and to determine simultaneously the number and size of both sporangia and zoospores. Full sporangia (no zoospore release), empty sporangia (zoospore release) and zoospores can be obtained in the same suspension after a time course experiment (Riemann et al., 2002). An automatic particle counter can therefore be used to count these propagules separately considering their size differences. Until now, this device has been successfully used to count and size oomycete sporangia (Flier et al., 2003; Clément et al., 2010; Peressotti et al., 2010a; Peressotti et al., 2010b; Delmotte et al., 2014).

We used a simple method to count and size sporangia and zoospores simultaneously in a biotrophic oomycete (Plasmopara viticola), the causal agent of grapevine downy mildew) and to investigate life-cycle trait correlations. An automatic particle analyzer was used to assess zoospore release dynamics in water. Manual and automatic counts and zoospore size data were compared to check the robustness of the automatic quantification of zoospores. This method made it possible to determine the number and the size of zoospores produced per sporangium and to study the variability of zoospore production in P. viticola populations. We also explored correlations between dispersal (sporangium number and size) and infection (zoospore number and size) potentials, two components of the pathogen transmission rate, in P. viticola.

2. Materials and methods

2.1. Plant material

We used Vitis vinifera cv. Cabernet Sauvignon, a cultivar that is grown worldwide and is known to be susceptible to downy mildew. The plants were grown in a greenhouse, under the natural photoperiod. For mildew amplification and experimental inoculation, the third and fourth leaves below the apex of young shoots were removed from the plants at the 10 unfolded leaves stage and rinsed with distilled water.

2.2. Pathogen isolates and leaf disk bioassay

In total, we studied 43 P. viticola isolates collected from infected leaves in vineyards in 2012 (see Table S1 in the supplementary content). Each isolate consisted of sporangia collected early in the season from a single sporulating lesion (oil spot) and stored in liquid nitrogen for further use.

The leaf bioassay involved the use of P. viticola isolates to inoculate cv. Cabernet Sauvignon leaf disks as previously described (Delmotte et al., 2014; Rouxel et al., 2013). The sporangial concentration of each isolate was adjusted to 10,000 sporangia/ml with a portable particle counter (Scepter 2.0TM automated cell counter; Millipore). For inoculation, four leaf disks per isolate were floated on the surface of the sporangial suspension, adaxial side up, for 4 h at 20 °C. The inoculated leaf disks were then placed abaxial side up in four square Petri dishes (23 × 23 cm) containing moistened filter paper (one leaf replicate per isolate per box). Petri dishes were sealed with cling film and placed in a phytotron (type LMS 610 XAP; LMS Ltd.; United Kingdom) with a 12 h (18 °C) light:12 h (15 °C) dark photoperiod, for seven days. For the purposes of this study, we considered the isolate to be the sampling unit.

Seven days after inoculation, we washed two disks per isolate separately, each in 15 ml of saline buffer (Isonit II, Coulter Corporation), to fix the pathogen cycle and collect any spores (t = 0 min; no zoospores released). The last two disks for each isolate were washed separately in 4 ml of distilled water, the resulting suspensions were kept at 20 °C to promote zoospore release and 11 ml of saline buffer was added at t = 100 min (some zoospores released). P. viticola sporangia and zoospores can be conserved in saline buffer at 4 °C until processing without spore lysis or encystment. Sporangium and zoospore quantification was not affected by Isonit dilution (C.E.L. Delmas, unpublished data).

2.3. Time course experiment with an automatic particle analyzer

We monitored zoospore release in one isolate (PV873) at six different time points (i.e. from full sporangia to complete zoospore release; Fig. 1), with an automatic particle analyzer. Seven days post-inoculation, an additional sporulating disk was washed in 100 ml distilled water. Three 2 ml samples were collected at t0 min, t30 min, t60 min, t90 min, t120 min and t150 min and each of these samples was added to 5.5 ml of saline buffer.

2.4. Automatic particle analyzer quantification and sizing

We automatically counted and sized particles at t0 min and t100 min for 43 P. viticola isolates. Five uninfected leaf disks were also rinsed in 15 ml of saline buffer and monitored with the particle analyzer to check for the absence of particles of debris of similar size to zoospores (control disks).

Samples were processed in a Multisizer 3 automatic particle counter (Coulter Counter® Multisizer™3; Beckman Coulter) as follows: particles suspended in the electrolyte solution are drawn through a small aperture (100 μm) separating two electrodes and displaced their own volume of electrolyte, momentarily increasing the impedance of the aperture. For each particle, the analyzer Multisizer 3 calculates a volume based on the extent of the change in impedance. It delivers a size (as 60 classes from 2 μm to 56.7 μm) corresponding to the diameter of a sphere having the same volume as the particle. Therefore, the diameter estimated does not correspond to the length or width of an ellipsoid sporangium (see Table 1 for actual manual sizing) but allows comparison of spore sizes between isolates. Reproducibility was insured by performing eight independent analyses of ten samples over a time period of one month.
2.5. Manual quantification and sizing under a stereomicroscope

We randomly chose one spore suspension (i.e. one sporulating leaf disk) per isolate for each monitored time point ($t_{0\text{ min}}$ and $t_{100\text{ min}}$; $N = 36$ and 43 isolates, respectively) to count the number of full sporangia (containing zoospores), empty sporangia (after zoospore release) and zoospores by eye, under a stereomicroscope ($\times 400$ magnification). We analyzed eight 1-μl drops of each spore suspension with a hemocytometer. In addition, we manually measured the size of 50 full sporangia, 50 empty sporangia and 50 zoospores under a stereomicroscope using a hemocytometer. These sporangia and zoospores were randomly chosen from the samples. Spore suspensions were stored for automatic particle analysis.

2.6. Data analysis

We calculated the mean numbers of sporangia and zoospores produced and the number of zoospores per sporangium for each isolate. The number of sporangia was estimated as the number of full sporangia in the $t_{0\text{ min}}$ samples (no zoospores release) and as the sum of full and empty sporangia in the $t_{100\text{ min}}$ samples (zoospore release). The number of zoospores per sporangium was estimated as the mean number of empty sporangia counted per sample (full sporangia were considered to have released no zoospores). The classification of zoospores, empty and full sporangia is based on particle size and was determined a posteriori from the three different size ranges evidenced in the result section (Fig. 2 and Fig. 3). We calculated sizes from the automatic particle analyzer results, as the weighted average of the particle size distribution for zoospores (between 2.5 and 5 μm) and empty sporangia (between 5 and 8 μm) at $t_{100\text{ min}}$ and for full sporangia (between 8 and 18 μm; including $t_{0\text{ min}}$ and $t_{100\text{ min}}$ samples). Mixed linear models (PROC GLIMMIX procedure in SAS software version 9.2) were used to compare the number of particles counted at $t_{0\text{ min}}$ and $t_{100\text{ min}}$ in the three size ranges. Time and size range effects and their interaction were treated as fixed factors and the isolate was the random factor. Particle counts were log-transformed to satisfy assumptions about normality and homoscedasticity. In addition, we assessed significant differences in particle number at $t_{0\text{ min}}$ and $t_{100\text{ min}}$ for each size range in least mean square tests (Tukey–Kramer tests adjusted for multiple comparisons).

Finally, Pearson’s correlation analysis was used to investigate the relationship between the numbers of particles counted with the two methods (manual counting with a hemocytometer and automatic counting with a particle analyzer) and to explore the phenotypic relationships between zoospore production, sporangium size and sporangium number.

3. Results

3.1. Time course experiment with an automatic particle analyzer

We first determined the numbers of sporangia and zoospores at six different time points for the P. viticola isolate PV873. We observed three different peaks in particle numbers over time. At $t_{0\text{ min}}$ and $t_{100\text{ min}}$ we detected a peak corresponding to full sporangia of between 8 and 18 μm in size (Fig. 2a). At $t_{100\text{ min}}$, the peak of full sporangia was still visible, but a new peak was beginning to emerge, corresponding to zoospores of 2.5 to 5 μm in diameter (Fig. 2b). At $t_{120\text{ min}}$ and $t_{150\text{ min}}$, full sporangia were no longer detected, there was a peak of empty sporangia of 5 to 8 μm in size and all the zoospores had been released (Fig. 2c,d).

Table 1

| Particle sizing with an automatic particle analyzer and a hemocytometer (manual sizing). For the automatic particle analyzer, particle size was calculated as the weighted average of the particle size distribution for zoospores (between 2.5 and 5 μm) and empty sporangia (between 5 and 8 μm) at $t = 100$ min and for full sporangia (between 8 and 18 μm; including $t_{0\text{ min}}$ and $t_{100\text{ min}}$ samples). Mixed linear models (PROC GLIMMIX procedure in SAS software version 9.2) were used to compare the number of particles counted at $t_{0\text{ min}}$ and $t_{100\text{ min}}$ in the three size ranges. Time and size range effects and their interaction were treated as fixed factors and the isolate was the random factor. Particle counts were log-transformed to satisfy assumptions about normality and homoscedasticity. In addition, we assessed significant differences in particle number at $t_{0\text{ min}}$ and $t_{100\text{ min}}$ for each size range in least mean square tests (Tukey–Kramer tests adjusted for multiple comparisons). Finally, Pearson’s correlation analysis was used to investigate the relationship between the numbers of particles counted with the two methods (manual counting with a hemocytometer and automatic counting with a particle analyzer) and to explore the phenotypic relationships between zoospore production, sporangium size and sporangium number.

3.2. Automatic particle analyzer quantification and sizing

We counted full sporangia, empty sporangia and zoospores at $t_{0\text{ min}}$ and $t_{100\text{ min}}$ over 43 P. viticola isolates based on the size ranges for these entities defined above (8 to 18 μm, 5 to 8 μm and 2.5 to 5 μm, respectively). At $t_{0\text{ min}}$, the relationship between particle number and size for all 43 isolates indicated that all the particles detected were full sporangia of about 11 μm in size (Fig. 3a; Table 1). At $t_{100\text{ min}}$, zoospores of about 3 μm in diameter were detected (Fig. 3a; Table 1). No zoospores were detected at $t_{0\text{ min}}$ or in control non-sporulating samples (Fig. 3a). Furthermore, as some, but not all the zoospores were released at $t_{100\text{ min}}$, two smaller peaks were detected in the average relationship between particle number and size over the 43 isolates: one corresponding to particles 5 to 8 μm in size (empty sporangia) and the other to particles 8 to 18 μm in size (full sporangia; Fig. 3a).

Thus, particle numbers differed significantly between $t_{0\text{ min}}$ and $t_{100\text{ min}}$ for the three size ranges (Table 2; Fig. 3b). There were larger numbers of zoospores and empty sporangia at $t_{100\text{ min}}$ than at $t_{0\text{ min}}$, whereas there were larger numbers of full sporangia at $t_{0\text{ min}}$ than at $t_{100\text{ min}}$.

The mean (±SD) number of sporangia produced per isolate was 6713 ± 2711 sporangia/ml, with a range of 1458 to 12,224 (summing empty and full sporangia). The mean number of zoospores was...
The mean number of zoospores per empty sporangium was 5.8 ± 1.9, with a range of 2.8 to 10.1 (mean = 3.5 ± 2.1/μl), and zoospores (mean = 11.27 ± 8.6/μl). At t = 100 min, we observed full sporangia (mean = 4.78 ± 2.19/μl), empty sporangia (mean = 11.27 ± 8.6/μl) in all samples. Empty and full sporangia were of similar size and the sporangia number = 5.95 ± 3.98 sporangia/100 μm. However, overall zoospore production increased significantly with sporangium production (Pearson r = 0.66; P < 0.0001; Fig. 5d). Finally, zoospore size was not significantly related to the number of zoospores per sporangium (Pearson r = −0.27; P = 0.07) or the number of zoospores produced (Pearson r = −0.12; P = 0.44).

4. Discussion

We used an automatic particle analyzer to quantify sporangia and zoospores simultaneously in P. viticola, the causal agent of grapevine downy mildew. By monitoring the dynamics of zoospore release, we
were able to identify well-separated peaks of particles corresponding to zoospores, full sporangia and sporangia that had released their zoospores. This was possible due to size differences between zoospores and sporangia and more importantly between full and empty sporangia. The empty sporangia collapsed during processing in the particle analyzer (see Fig. 1) and were therefore found to be smaller than full sporangia. Although the particle analyzer discriminated between particles of different sizes, it did not provide actual size estimates since particle diameter estimations are based on particle volumes and also due to the deformation of empty particles during the sizing process. The significant correlation between automatic and manual sporangium and zoospore quantification demonstrated the robustness of the automatic method. In addition, the ratio of full to empty sporangia at a given time point may provide an indirect measurement of the temporal dynamics of zoospore release for isolates.

The use of a particle analyzer made it possible to estimate mean sporangium and zoospore production by *P. viticola* for 43 isolates, together with the mean number of zoospores produced per sporangium (5.7 zoospores of about 5 μm in diameter per sporangium). The number of zoospores released per sporangium is known to vary between species (Walker and West, 2007), but little data have been published concerning this aspect in most oomycetes. The counting time required for a 500 μl sample is about 15 s and the numbers of sporangia and/or zoospores counted per sample are two to three orders of magnitude greater than the number of spores counted by eye under a stereomicroscope; the use of a particle counter thus increases statistical reliability and measurement accuracy (Schoulties and Yang, 1970). Furthermore, the use of a particle analyzer makes it possible to investigate the large numbers of samples (i.e. pathogen isolates), a key feature for population studies.

In this study, we were able to quantify the potentials for both dispersal (i.e. sporangial production) and infection (i.e. the number of zoospores produced by sporangia and overall zoospore production) in a biotrophic oomycete. These two potentials are components of the rate of transmission (Lannou, 2012). We demonstrated the existence of a negative relationship between sporangium size and the number of sporangia produced, confirming the findings of Delmotte et al. (2014). These results suggest that *P. viticola* isolates producing large numbers of small sporangia in secondary infections probably have a higher dispersal potential, because the sporangia of these isolates have more chance of reaching new healthy host tissue. Delmotte et al. (2014) suggested that this enhanced capacity for dispersal may play a

![Fig. 4. Correlation between the number of zoospores per ml determined with an automatic particle analyzer and that determined with a hemocytometer under a stereomicroscope (Pearson r = 0.79; P < 0.0001). Automatic and manual zoospore counts were obtained for the same spore suspension for each isolate, at t = 100 min (N = 43).](image)

![Fig. 5. Phenotypic correlations between (a) mean number of sporangia produced and sporangium size; (b) mean number of zoospores produced per sporangium and sporangium size; (c) mean number of zoospores produced per sporangium and sporangium production; and (d) mean number of zoospores produced and sporangium production for 43 *Plasmopara viticola* isolates.](image)
key role in the colonization of new habitats by aggressive isolates. However, it remains unknown whether such small sporangia have the same infection potential as larger sporangia, although this factor is known to be a key driver of pathogen population evolution. Our investigations of zoospore production revealed that the number of zoospores per sporangium was related to sporangium size, with smaller sporangia producing fewer zoospores. Consequently, in aggressive isolates, which were characterized by many small sporangia, each sporangium produced fewer zoospores, decreasing the infection potential. However, total zoospore production increased with sporangium production. This pattern should counteract the relationship between sporangium size and the number of zoospores produced per sporangium, conferring a fitness advantage on aggressive isolates producing large numbers of small sporangia.

The existence of several tradeoffs in plant pathogens has been reported, including negative relationships between transmission (spore production) and a) aggressiveness (Anderson and May, 1982; Frank, 1996; Koella and Agnew, 1999; Paul et al., 2004), b) duration of the latent period (Héraudet et al., 2008; Pariaud et al., 2013) and c) virulence (Thrall and Burdon, 2003). A negative relationship between offspring number and size has been reported for both animals and plants and is a classic example of a trade-off between life-history traits (Stearns, 1992; Roff, 2002). To our knowledge, the negative relationship between sporangium size and production highlighted here for P. viticola is the first report of such a correlation in a plant pathogen species (Delmotte et al., 2014). Moreover, the negative phenotypic correlation between dispersal and infection potentials reported here suggests that there may be a phenotypic tradeoff between these two life-history traits. Genetic correlations between these traits should be investigated, to shed light on possible evolutionary constraints due to physiological or micro-evolutionary tradeoffs (Stearns, 1992; Roff, 2002; Héraudet et al., 2008). Such phenotypic trade-offs may contribute to the maintenance of genetic variation in sporangium production, sporangium size and the number of zoospores produced by sporangia, all of which are crucial life-history traits in oomycetes.

5. Conclusion
We simultaneously quantified and sized sporangia and zoospores in a biotrophic oomycete. This simple method, making it possible to process large numbers of samples, can be used to estimate two factors underlying the rate of transmission of phytopathogenic oomycetes: dispersal potential (i.e. sporangium production) and infection potential (i.e. zoospore production). Our results confirm the existence of a negative relationship between sporangium size and sporangium production and provide the first demonstration of a phenotypic correlation between sporangium size and the number of zoospores per sporangium. This negative correlation between dispersal and infection potentials at the sporangium scale highlights the need to estimate both these traits together when considering the evolution of plant pathogens.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2014.10.012.

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