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TECHNICAL ADVANCE

Phytophthora infestans Sporangia Produced in Artificial Media and Plant Lesions Have Subtly Divergent Transcription Profiles but Equivalent Infection Potential and Aggressiveness

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Sporangia of the potato late blight agent *Phytophthora infestans* are often used in studies of pathogen biology and plant responses to infection. Investigations of spore biology can be challenging in oomycetes because their sporangia are physiologically active and change in response to environmental factors and aging. Whether sporangia from artificial media and plant lesions are functionally equivalent has been a topic of debate. To address these issues, we compared the transcriptomes and infection ability of sporangia from rye-sucrose media, potato and tomato leaflets, and potato tubers. Small differences were observed between the mRNA profiles of sporangia from all sources, including variation in genes encoding metabolic enzymes, cell-wall-degrading enzymes, and ABC transporters. Small differences in sporangia age also resulted in variation in the transcriptome. Taking care to use sporangia of similar maturity, we observed that those sourced from media or plant lesions had similar rates of zoospore release and cyst germination. There were also no differences in infection rates or aggressiveness on leaflets, based on single-spore inoculation assays. Such results are discordant with those of a recent publication in this journal. Nevertheless, we conclude that sporangia from plant and media cultures are functionally similar and emphasize the importance of using "best practices" in experiments with sporangia to obtain reliable results.

Keywords: oomycete–plant interactions, Phytophthora diseases.

Data availability: Fastq files used for RNA-seq analysis are deposited in the NCBI Short Read Archive as accessions SAMN10601059 to SAMN10601082.

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Asexual sporangia are made by most oomycete phytopathogens and play central roles in disease. Sporangia travel by wind or water to disseminate the pathogen and initiate infections (Leesutthiphonchai et al. 2018). Unlike conidia of most fungi, which are desiccated, oomycete sporangia are hydrated and physiologically active. This enhances infection potential by enabling sporangia to respond to germination-conducive conditions. For example, exposing sporangia to water triggers zoosporogenesis, in which major cytoplasmic and transcriptomic changes occur within minutes of the stimulus (Tani et al. 2004; Walker and van West 2007). The fact that sporangia are not desiccated makes them prone to damage from laboratory manipulations as well as natural environmental factors such as heat or light (Mizubuti et al. 2000). The ephemeral nature of sporangia and their rapid responses to environmental signals can make the acquisition of highly reproducible experimental results a challenge.

Many laboratories use sporangia produced in artificial media for studying oomycete biology, assessing the effectiveness of plant resistance genes, and characterizing host responses to infection. For example, we and others have employed cultures grown on a rye grain-based medium to study sporulation, germination, zoospore motility, appressorium formation, and host colonization by wild-type and gene knock-down strains of *Phytophthora infestans*, the potato late blight pathogen (Ah-Fong et al. 2017a; Belhaj et al. 2017; Boevink et al. 2016; Gamboa-Meléndez et al. 2013; Haas et al. 2009; Latijnhouwers et al. 2004; Leesutthiphonchai and Judelson 2018). Sporangia from artificial media are also commonly used in assays of host resistance (Kirk et al. 2005; Naess et al. 2000). However, many researchers prefer to use sporangia produced on host tissue for experiments (Bradshaw et al. 2006; Foolad et al. 2014). Passage through the host may help eliminate deleterious mutations or establish epigenetic states that contribute to fitness. There may also be physiological differences between sporangia from plants and artificial media. Oomycete hyphae lack septa and their cytoplasm flows into sporangial initials during sporulation (Hardham and Hyde 1997). Therefore, proteins and mRNAs made during plant colonization may be deposited in sporangia, where they may later contribute to pathogenesis. Many defense-suppressing effectors and cell-wall-degrading enzymes are expressed by *P. infestans* primarily during plant infection (Ah-Fong et al. 2017b; Haas et al. 2009).

It was suggested recently that differences between *P. infestans* sporangia from artificial media compared with

plant lesions may explain why some crop protection strategies that initially looked promising in laboratory assays failed upon further testing (Fry 2016). To explore that concept and test the validity of past research on *P. infestans* spore biology, we compared the transcriptomes and infection potential of sporangia produced on artificial media, tomato and potato leaflets, and potato tubers. Taking precautions to ensure that the sporangia from each source were of equal age and harvested carefully, we found that the origin of sporangia had only subtle effects on their transcriptomes. We failed to observe differences in the abilities of sporangia generated in artificial media or in planta to cause infection. Our results conflict with those reported in a recent study by another laboratory (Fry et al. 2019).

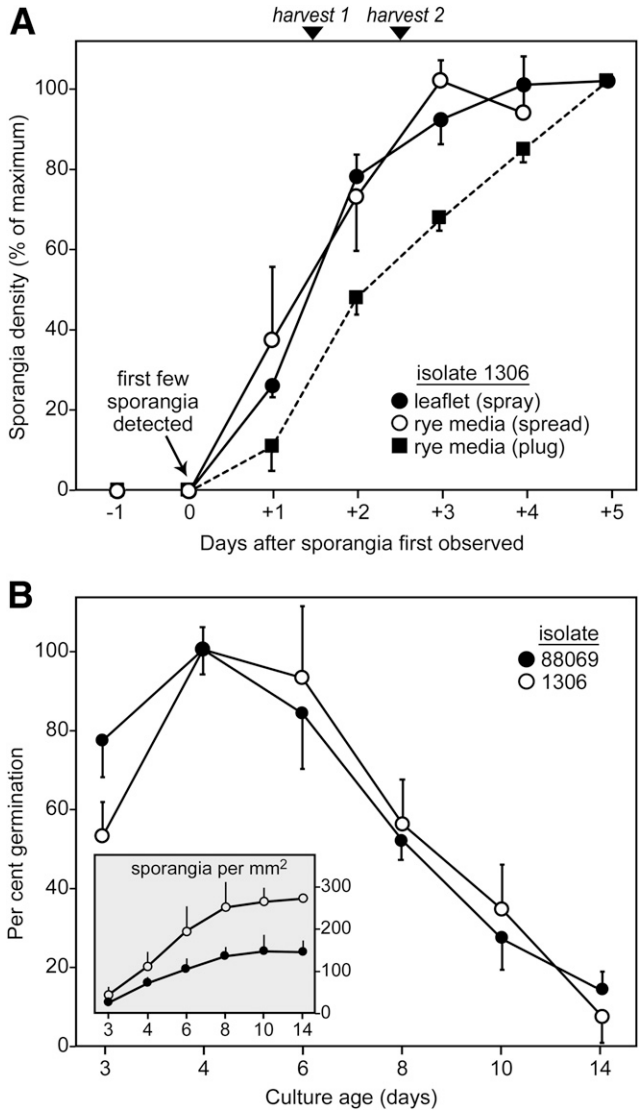

Fig. 1. Influence of age on sporulation and germination in *Phytophthora infestans*. **A,** Time-course of sporulation using isolate 1306. Sporangia were harvested from spray-inoculated tomato leaflets (black circles), rye-sucrose agar inoculated with a lawn of sporangia (open circles), or rye-sucrose agar inoculated with a plug (black squares). Time 0 is when a few sparse sporangia were first observed on leaflets or agar plates. Values are expressed as the percentage of maximum, which equaled 710 ± 69, 247 ± 25, and 331 ± 56 sporangia/mm^2 for the infected leaflet (day +5), spread-inoculated rye media (day +3), and plug-inoculated rye media samples (day +5), respectively. Error bars represent standard deviation of biological replicates. **B,** Effect of culture age on indirect germination. Indicated are the fractions of sporangia that had released zoospores 3 h after being placed in Petri's solution at 10°C. Values are expressed relative to the maximum for each isolate, which equaled 82 and 71% germination for isolates 88069 and 1306, respectively, on day 4. Direct germination was not observed under the conditions of the experiment. The inset graph shows the density of sporangia at each timepoint in the cultures, which were inoculated with plugs.

RESULTS

Strategy for collecting and handling sporangia.

We believe that sporangia need to be treated with special care to allow their infection potential, transcriptome content, and other characteristics to be assessed accurately. This applies when comparing sporangia from cultures grown under different conditions or a parental strain with transformants that overexpress or are silenced for a gene of interest. Although our detailed protocols for manipulating sporangia are documented in Materials and Methods, an overview is presented in the following paragraphs.

Our preferred method for obtaining sporangia from artificial media is to start by inoculating the entire surface of a rye-sucrose culture plate with sporangia. This leads to uniform hyphal growth, followed by sporulation. Depending on the amount of inoculum and incubation temperature (typically 18°C), we start to observe a tiny number of new sporangia after approximately 5 days, which is recorded as day 0 in Figure 1A. Sporulation plateaus on day +3, with 70% of sporangia produced on days +1 and +2. The kinetics of sporulation obtained with this protocol match closely that obtained with zoospore-sprayed tomato leaflets, where most sporangia also appear on days +1 and +2 (Fig. 1A). These data were obtained using isolate 1306 but other isolates have yielded similar results.

Based on the literature, most laboratories obtain sporangia from plates inoculated with a plug from a stock culture. Cultures grown in this matter accrue sporangia over at least 6 days (Fig. 1A, dashed line). Many articles describe collecting sporangia after 10 to 14 days; the sporangia would thus range from approximately 1 to 10 days in age.

That age is an important variable is illustrated in Figure 1B, which shows the relative fraction of sporangia releasing zoospores (i.e., indirect germination) in isolates 1306 and 88069 after 3 h at 10°C. Germination was highest in sporangia from cultures 4 to 5 days after inoculation but declined dramatically by day 14. We frequently observe up to 90% germination, which is similar to that reported for *P. infestans* by many other laboratories (Elsner et al. 1970; Latijnhouwers et al. 2004). It should be noted that germination rates in the literature may vary due to many factors, including the incubation conditions and when germination is assessed. Nevertheless, other researchers have also reported that zoospore release decreases in cultures that have aged over a time period similar to that shown in Figure 1B (King et al. 1968).

We paid close attention to the methods used to harvest sporangia. We typically collect sporangia in a modified version of Petri's solution (0.25 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4, and 0.8 mM KCl; unadjusted pH is 4.9), which was first developed to stimulate sporulation and zoospore release from *P. cambivora* (Petri 1917). Sporangia are also diluted in this buffer when equalizing their concentrations for germination and infection assays. We have found that the use of the buffer reduces batch-to-batch variation in germination, possibly by equalizing pH or replenishing ions that leach from sporangia during harvesting. Dew on leaf surfaces is typically slightly acidic, and ion levels as well as pH both affect zoospore release (Byrt and Grant 1979; Halsall and Forrester 1977; Sato 1994b; Vanbruggen et al. 1987).

The swift harvesting of sporangia is also important because changes begin when they are placed in water. It may take 60 min for cytoplasmic reorganization to be visually evident but

many zoosporogenesis-associated genes are induced within 20 min of exposure to cool water (Tani et al. 2004). Placing sporangia in water at room temperature also causes the abundance of some mRNAs to rise rapidly and others to fall (Tani et al. 2004). Our past attempts to block such changes by harvesting sporangia in high concentrations of actinomycin D (100 µg/ml) had only limited success, possibly because this transcriptional inhibitor slowly crosses the sporangial wall (Judelson and Roberts 2002). Instead, we rushed to harvest each batch of sporangia within 8 to 10 min. For RNA or protein analysis, this means that sporangia are washed from leaf or culture plate surfaces, filtered to remove contaminating hyphae, concentrated by centrifugation, and flash-frozen within 10 min. Some researchers describe harvesting *P. infestans* sporangia by centrifugation at very high speeds (Fry et al. 2019) but we use the minimum possible (700 × *g*) because even moderate *g*-forces trigger rapid changes in most eukaryotic cells (Soto et al. 2007).

Transcriptomes are influenced modestly by the source of sporangia.

We employed RNA-seq to compare sporangia from rye media, potato leaflets, tomato leaflets, and potato tuber slices using isolate 1306, with three biological replicates. We were careful to use sporangia of similar age and to harvest the sporangia rapidly. Samples were obtained from time points matching "harvest 1" and "harvest 2" in Figure 1A in order to represent sporangia on the first and second major days of sporulation. This corresponded to 4.5 and 5.5 days postinfection on plant tissue, and 6.5 and 7.5 days on rye-sucrose media. The sporangia were passed through a 50-µm mesh filter to eliminate hyphal fragments that may have been dislodged from the cultures. A minimum of 25 million 75-nucleotide (nt) single-end reads were then obtained from each replicate (Supplementary Table S1). Principal component analysis (PCA) identified fairly tight clustering of the replicates, with the least variation seen between those from rye-sucrose media (Fig. 2B).

We identified modest differences in mRNA profiles attributable to both the source of sporangia and the harvest date. This and subsequent analyses were limited to the 15,522 genes having counts per million mapped reads (CPM) > 1.0 in at least one condition. When pairwise comparisons were made of sporangia from rye-sucrose media, potato leaflets, tomato leaflets, or potato tubers, an average of 804 genes or 5.2% of the total exhibited >twofold differences based on a Benjamini-Hochberg false discovery rate (FDR) threshold of 0.05. This variation is evident in the heatmap in Figure 2A and enumerated in Figure 2C. The fewest differences were between sporangia from potato and tomato leaflets. Expression values for the genes and differential expression statistics are shown in Supplementary Tables S2 and S3.

Although growth conditions (e.g., media versus leaflets) contributed most to the differences, age was also a factor. Although little variation was observed between the harvest 1 and

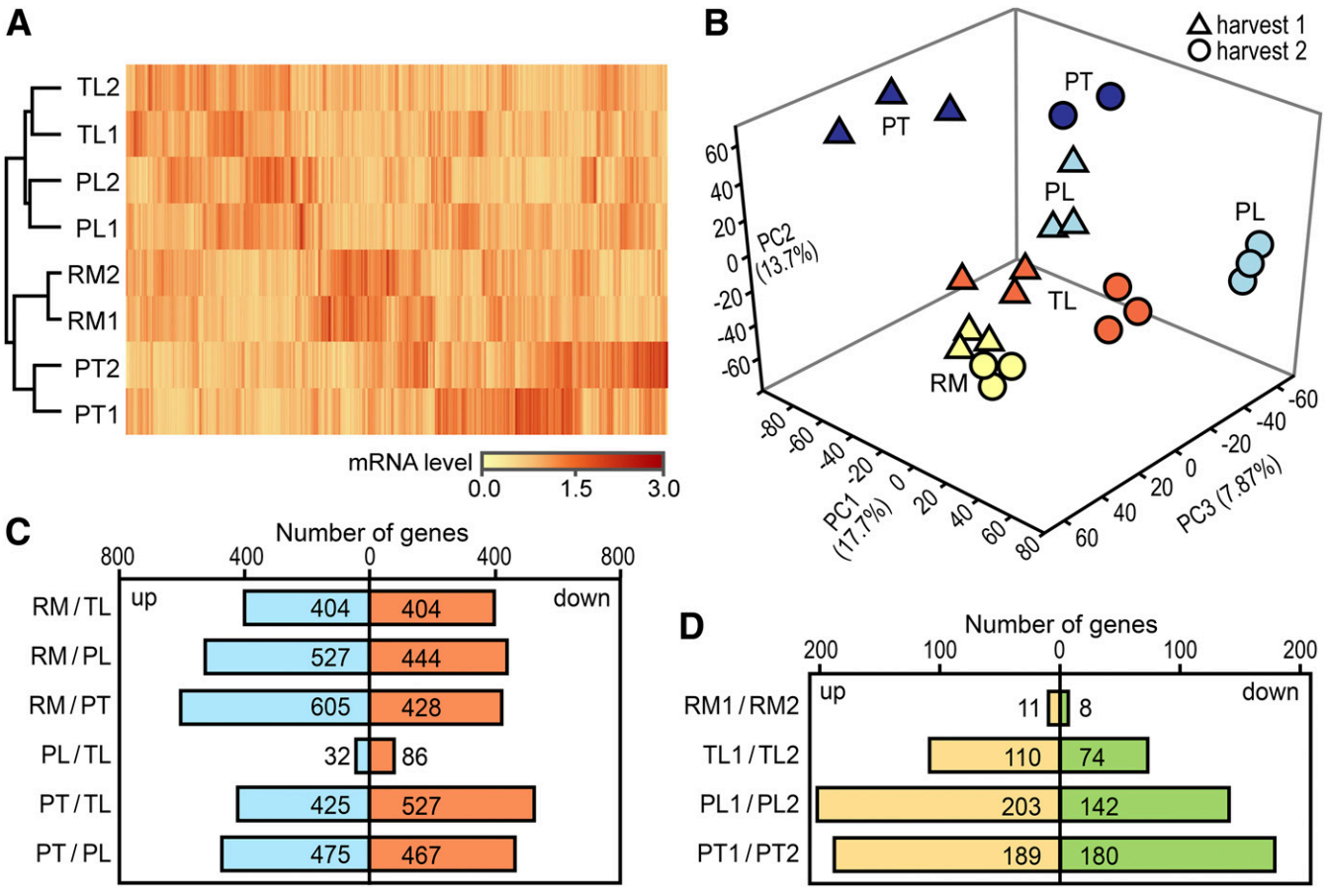

Fig. 2. mRNA levels in sporangia from different culture conditions. **A,** Heatmap representing mRNA from tomato leaflets (TL), potato leaflets (PL), rye-sucrose media (RM), and potato tubers (PT). Samples were taken at the harvest 1 and harvest 2 timepoints, representing sporangia on the first (e.g., TL1) and second (e.g., TL2) major days of sporulation. **B,** Principal component analysis of the RNA samples. Each symbol represents a biological replicate. **C,** Number of genes that were differentially expressed between culture conditions, based on thresholds of >twofold change and false discovery rate < 0.05 at the two timepoints. **D,** Number of genes differentially expressed between sporangia from the harvest 1 and harvest 2 timepoints; these timepoints correspond to 4.5 and 5.5 days postinfection on plant tissue and 6.5 and 7.5 days on rye-sucrose media.

harvest 2 sporangia from rye media, an average of 316 genes exhibited >twofold differences between timepoints in one or more of the leaflet and tuber samples (Fig. 2D). That differences would arise between the two timepoints is unsurprising because sporulation in planta occurs during the transition of *P. infestans* from biotrophy to necrotrophy, which would presumably alter the host environment. More age-specific differences were observed in the potato compared with tomato infections, which may reflect the fact that host necrosis with isolate 1306 is typically more extensive in potato than tomato.

The RNA-seq data were also used to scrutinize the quality of the sporangia samples. One check was to ensure that zoosporogenesis-specific genes had not been induced. This might have occurred if our harvesting protocol was slow, resulting in false positives in the differential expression studies. This inspection used several genes, including transporter gene PITG_13579, which is induced rapidly when sporangia are placed in water (Fig. 3A). The two left-most lanes in Figure 3B show a similar experiment using RNA-seq, where chilling increased the fragments per kilobase of exon per million mapped reads (FPKM) value by at least 100-fold. The other lanes represent the 24 sporangia samples from rye media, potato and tomato leaflets, and potato tubers. None expressed much PITG_13579 mRNA, and similar results were obtained with other zoosporogenesis-induced genes, including PITG_13496, PITG_09169, and PITG_13170. This indicates that the transcriptomes of the 24 sporangia samples are representative of proper ungerminated sporangia, and helps confirm the fidelity of the differential expression data in Figure 2 and our supplementary tables.

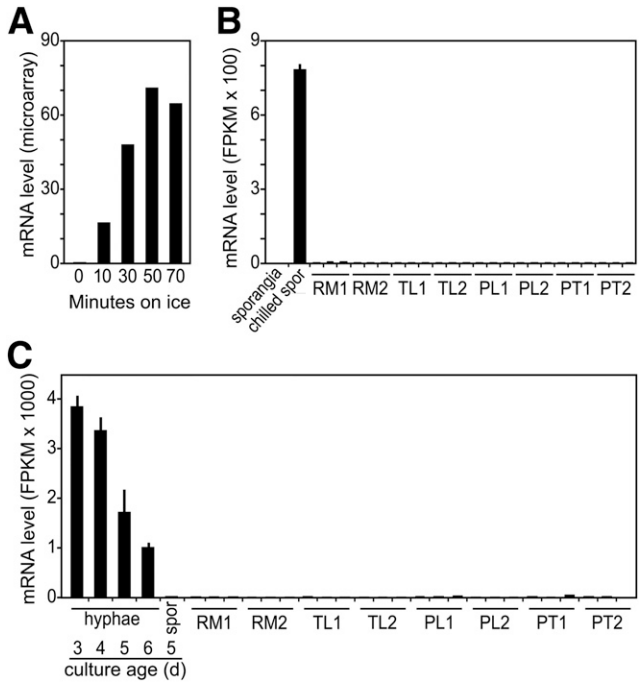

Fig. 3. Quality assessments of the 24 sporangia samples. **A,** Expression of zoosporogenesis-induced gene PITG_13579 in sporangia immediately after harvesting and after 10, 30, 50, and 70 min of chilling in modified Petri's solution. Data are based on microarray analysis. **B,** Abundance of PITG_13579 mRNA based on RNA-seq. The two left samples are freshly harvested sporangia and the same chilled for 0 and 60 min (chilled spor.). Samples labeled RM, TL, PL, and PT are sporangia from rye media, tomato leaflets, potato leaflets, and potato tubers, respectively, from harvest 1 and harvest 2; these timepoints correspond to 4.5 and 5.5 days postinfection on plant tissue and 6.5 and 7.5 days on rye-sucrose media. **C,** Abundance of PITG_21410 mRNA based on RNA-seq. The left side of the panel illustrates expression of the gene in hyphae collected from cultures between 3 and 6 days after inoculation, and sporangia from a 5-day culture. The right side of the panel shows expression in the 24 RM, TL, PL, and PT sporangia samples.

A second quality-control check examined genes that are normally expressed at high levels during hyphal growth but are repressed during sporulation. If sporangia were contaminated with hyphal fragments or sporangiophores, then these genes would show a substantial signal in the RNA-seq data. In addition, if some samples had much higher signals than the others, that might mean that we had mistimed the two harvests. One gene used for this analysis was PITG_21410, which encodes elicitin-like protein INF4 (Jiang et al. 2006). The mRNA level of this gene declines in sporulating cultures and is very low in sporangia (Fig. 3C, left side). All 24 rye media, leaflet, and tuber samples exhibited very low levels of expression of this gene (Fig. 3C, right side). Similar results were obtained with other sporulation-repressed genes such as PITG_09454 and PITG_12556. The finding that the signal was low in all samples supports the fidelity of the comparisons in Figure 2 and our supplementary tables.

Metabolic functions predominate among differentially expressed genes.

Gene ontology (GO term) analyses associated a limited number of functional categories with genes that were differentially expressed between sporangia sourced from media and any of the three types of plant lesions. Many of the differences between the plant and rye-sucrose media samples were related to metabolism. For example, overrepresented GO terms included oxidoreductase activity (GO:0016491, *P* adjusted [*Padj*] = 9e-4), tyrosine catabolism (GO:0006572, *Padj* = 7e-4), and glycosyl hydrolase activity (GO:0016798, *Padj* = 3e-2). A full list is shown in Supplementary Table S4.

Notable differences were seen for genes encoding enzymes that degrade plant cell walls (Fig. 4A). For example, the aggregate level (summed FPKM) of cutinase transcripts was much lower in rye-sucrose media and tubers than the two leaflet samples, which is logical because only the leaflets contain appreciable amounts of cutin. Also logical is our observation that sporangia from rye media contained higher levels of xylosidase mRNA, because rye grain is much richer in xylans than potato or tomato (Blaschek et al. 1981; Jarvis et al. 1981; Knudsen 2014).

A broader analysis of metabolism based on KEGG pathways (Aoki-Kinoshita and Kanehisa 2007) indicated that sporangia from rye-sucrose media clustered separately from the plant samples, with the potato and tomato leaflet samples clustering apart from tubers (Fig. 4B). This pattern had also been observed with total genes (Fig. 2A). Most pathways related to carbohydrate metabolism such as glycolysis, the TCA cycle, and the pentose phosphate pathway had higher aggregate mRNA levels in sporangia from rye-sucrose media than from leaflets or tubers. This is likely because the concentration of soluble sugars is approximately five times higher in the rye-sucrose media (Judelson et al. 2009). There were also differences in other pathways, including cofactor, fatty acid, and amino acid metabolism. Several transporters were also differentially expressed such as nitrate transporters (PITG_09342 and PITG_25173) which exhibited approximately three times higher transcript levels on leaflets than rye media or tubers. This may be attributable to the fact that our plants were fertilized with nitrate, which is at low levels in tubers and rye-sucrose media (Abrahamian et al. 2016).

An analysis of genes traditionally associated with pathogenesis revealed that ABC transporters tended to have higher mRNA levels in sporangia from leaflets compared with rye-sucrose media. Such proteins are thought to participate in the efflux of toxins, including plant defense molecules (Perlin et al.

2014). The ratio of mRNA abundance in sporangia harvested from tomato leaflets versus rye-sucrose media was skewed toward higher expression in the leaflets (Fig. 4C), with average expression being 23% higher in the leaflets. Based on a *P* value threshold of 0.01, 22 ABC transporter genes had higher transcript levels in sporangia from tomato leaflets compared with artificial media. Similar results were obtained for sporangia from potato leaflets, while mRNA levels of ABC transporters in sporangia from media and potato tubers were similar. Other genes potentially used to defend against chemical toxicants, such as those encoding catalases and peroxidases, were expressed at similar levels in all samples.

Few genes encoding RXLR proteins exhibited significant differential expression between the types of sporangia, based on analysis of the 200 genes that had CPM > 1 in any sample. A volcano plot of their transcript levels in tomato leaflets versus rye-sucrose media showed only three with higher mRNA in the leaflets at a $P < 0.01$ threshold (Fig. 4D). Only two RXLRs that were infection induced, PITG_05846 and PITG_06375, had higher mRNA in each of the three types of plant-derived sporangia compared with rye-sucrose. The horizontal dispersion of RXLR signals in the plot was wider than that of ABC transporters because many RXLR genes are up- or downregulated during sporulation, leading to greater statistical noise (Ah-Fong et al. 2017a).

Expressed at CPM > 1 in sporangia were 114 CRN genes. However, none of their mRNA levels were consistently higher in sporangia from a plant source compared with rye-sucrose media, although PITG_19565 was twofold higher in potato leaflets compared with media. Instead, CRN genes were more likely to exhibit higher mRNA levels in sporangia from media.

Transcript levels of genes associated with sporulation varied little between sporangia sourced from plants and media. This conclusion is based on the analysis of genes that encode proteins specific to the flagellar axoneme (Judelson et al. 2012). This is expected because inhibitor studies demonstrated that most zoospore proteins are preformed in sporangia and not translated during germination (Clark et al. 1978). Due to normal mRNA decay in aging sporangia, it was also not surprising that the transcript levels of most flagella-associated genes were lower in harvest 2 versus the harvest 1 sporangia, because the latter tend to be younger. For example, genes encoding intraflagellar transport, basal body, and mastigoneme proteins exhibited median declines in mRNA of 7, 15, and 29%, respectively, between the timepoints. This decline highlights the importance of using sporangia of similar age in critical experiments. A prior study identified much greater differences in the mRNA levels of flagellar protein genes in sporangia from plants 6 days after infection than from artificial media cultures 12 days after inoculation (Fry et al. 2019). Although that study

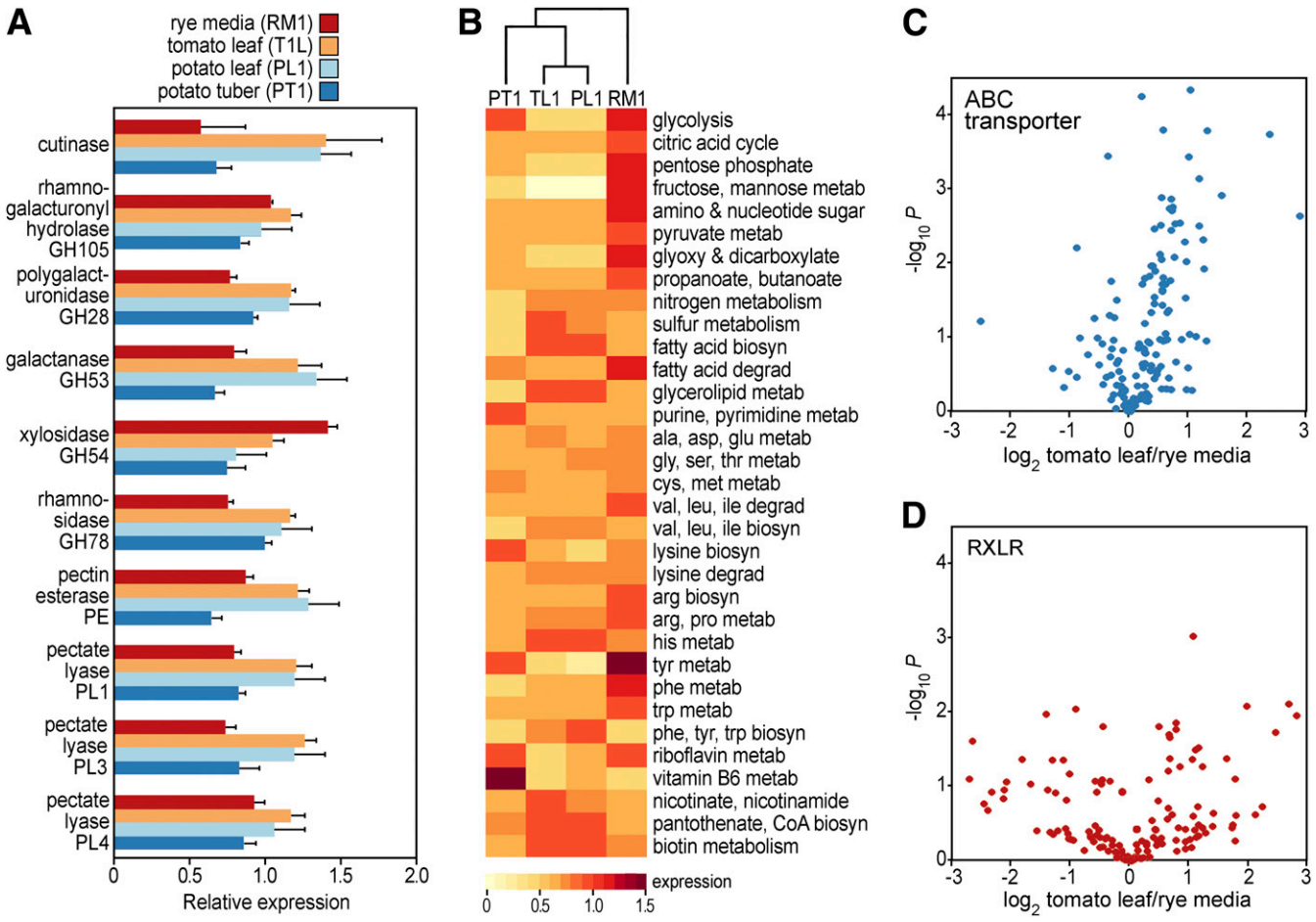

Fig. 4. Effect of sporangia source on mRNA levels of selected functional groups. **A,** Cell-wall-degrading enzymes. Sporangia were from harvest 1 tomato leaflets (TL1), potato leaflets (PL1), rye-sucrose media (RM1), and potato tubers (PT1). Fragments per kilobase of exon per million mapped reads levels of genes in each group were added together and normalized to the average of the group. Results from the harvest 2 sporangia (collected 5.5 days postinfection on plant tissue and 7.5 days postinoculation on rye-sucrose media) were similar. **B,** Metabolic pathways. Samples are the same as those in A, and expression levels were normalized to the average of each functional category. **C,** ABC transporters. Indicated are the ratios of mRNA for each gene in sporangia from tomato leaflets (TL1) versus rye media (RM1). **D,** Same as C but for RXLRs.

concluded that this meant that plant-sourced sporangia are more primed for indirect germination, our interpretation of their result is that it provides further proof of the importance of controlling for sporangia age in such experiments.

Sporangia size varies with their origin.

The dimensions of *P. infestans* sporangia differed depending on their source. In particular, those generated on rye-sucrose media tended to be larger than those from potato or tomato leaflets, although the length/breadth ratio was not significantly different (Fig. 5). That growth conditions affect sporangial geometry has been reported previously for other members of the genus (Brasier and Griffin 1979). Based on concepts derived from mathematical modeling of fungal growth (Lew 2011), our observations suggest either that extensibility of the sporangial wall is greater on rye media compared with on plants or that cytoplasm flows faster into sporangial initials formed on media.

Spores from all sources have the same infection potential.

The dissimilarities in the shapes and transcriptomes of sporangia from media and plant lesions were modest but might signal that propagules from different sources would vary in their infection ability. This was tested by comparing sporangia of isolate 1306 from rye-sucrose media with those from lesions on potato leaflets. To begin, sporangia of the same age were harvested from the two sources in parallel. Indirect germination was then stimulated using cold modified Petri's solution. After 3 h, zoospores were separated from sporangia by filtration through 15-µm mesh and diluted to 100 zoospores/ml, and individual leaflets were inoculated with a single drop bearing 1 zoospore. This was repeated in three separate experiments, using 100 leaflets per treatment per experiment, which after inoculated leaflets were positioned in a plant growth chamber using a randomized complete block design. Materials were rotated daily to ensure that the leaflets were exposed to equivalent light and humidity.

The results from these single-zoospore infection assays indicated that the infection potential of spores from plants and artificial media were equivalent (Fig. 6A and B). There was no significant difference based on one-way analysis of variance and Wilcoxon signed-rank tests. The average infection potential of each zoospore was 32%, which is in the range described for *P. nicotianae* (Kong and Hong 2016). The average infection rate varied in our single-zoospore experiments from 10 to 58%, which we believe is due to differences in the age of the plants. Infection assays performed using five zoospores per inoculum drop also resulted in similar rates of infection by the media and leaflet-sourced spores, although these experiments were less informative because close to 100% of leaflets were often infected.

The source of spores also had no significant effect on the pace of lesion expansion. This conclusion is based on measurements taken between 5 and 8 days postinfection in the three single-zoospore experiments (Fig. 6C). Moreover, the total amount of sporangia produced on the leaflets by day 8 was similar regardless of whether the inoculum was from leaflets or artificial media (Fig. 6D). Therefore, similar aggressiveness was exhibited by sporangia from both sources.

Because foliage infections are usually initiated by sporangia, we also attempted to assess whether sporangia from plants and rye-sucrose media had similar infection potentials. This involved placing a droplet of water containing a single sporangium on each of multiple leaflets, which were placed at 10°C for 4 h to trigger zoosporogenesis and then incubated at 18°C in a plant growth chamber. Excluding cases where the inoculum drop appeared to have run off the leaflet, close to 100% of leaflets became infected. Because the rate of colonization was so high, we cannot exclude the possibility that miniscule differences may exist in the infection potential of sporangia from plants and media but such small differences may not be biologically relevant.

Sporangia from plants and artificial media germinate at similar rates.

Although the rates of zoosporogenesis of the two types of sporangia were not quantified precisely in the plant infection experiments described above, they appeared similar based on microscopic examination. Several additional studies confirmed that zoosporogenesis progresses similarly in plant and media-sourced sporangia.

In the first experiment, we quantified zoospore release from isolates 1306 and 88069. This used sporangia that had been harvested from tomato leaflets and media 6 days after inoculation. With both strains, the source of sporangia had little if any effect on germination based on observations made 2 and 12 h after chilling the spores (Fig. 7A). For example, after 12 h, 76 ± 10 and 69 ± 5% of sporangia from isolate 1306 sourced from tomato leaflets and rye-sucrose media had germinated, respectively. Because zoospores from plant and media were shown above to have similar infection potentials, it follows that the same should hold for sporangia.

A second study also compared the germination of sporangia obtained from tomato leaflets, potato leaflets, and media, this time using only isolate 1306 (Fig. 7B). This experiment also evaluated sporangia harvested 5, 6, and 8 days after inoculation. Based on observations made 3 and 6 h after chilling, the source of sporangia had little effect on germination. This provides further support to the hypothesis that growing *P. infestans* on a plant does confer upon its sporangia a higher infection potential. It is interesting that the 5- and 8-day sporangia germinated at similar rates in this study, whereas germination was impaired in the older cultures in the Figure 1B experiment. We believe that this is because the cultures in Figure 1B were plug inoculated, whereas those in Figure 7B were initiated by spreading sporangia. Because hyphal growth is denser in plug-inoculated cultures, sporangia germination may be influenced by concentrations of nutrients or waste products in the underlying media. This observation stresses the importance of preparing sporangia under standardized conditions.

We also measured the germination of cysts derived from the plant- and media-sourced zoospores used in the infection assays shown in Figure 6. This involved placing aliquots of the zoospores in clarified rye-sucrose broth to trigger encystment and germination. The cyst germination rates in the three experiments in Figure 6 averaged 69 and 78% for sporangia from

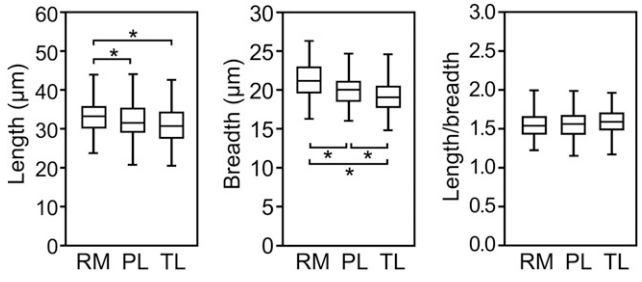

Fig. 5. Sporangia shapes from rye-sucrose media (RM), potato leaflets (PL), and tomato leaflets (TL). Values are based on a minimum of 200 sporangia per sample at the harvest 1 timepoint (4.5 days postinfection on plant tissue and 6.5 days postinoculation on media). Variation was assessed using a paired *t* test, and pairs differing at $P < 0.01$ are marked by an asterisk.

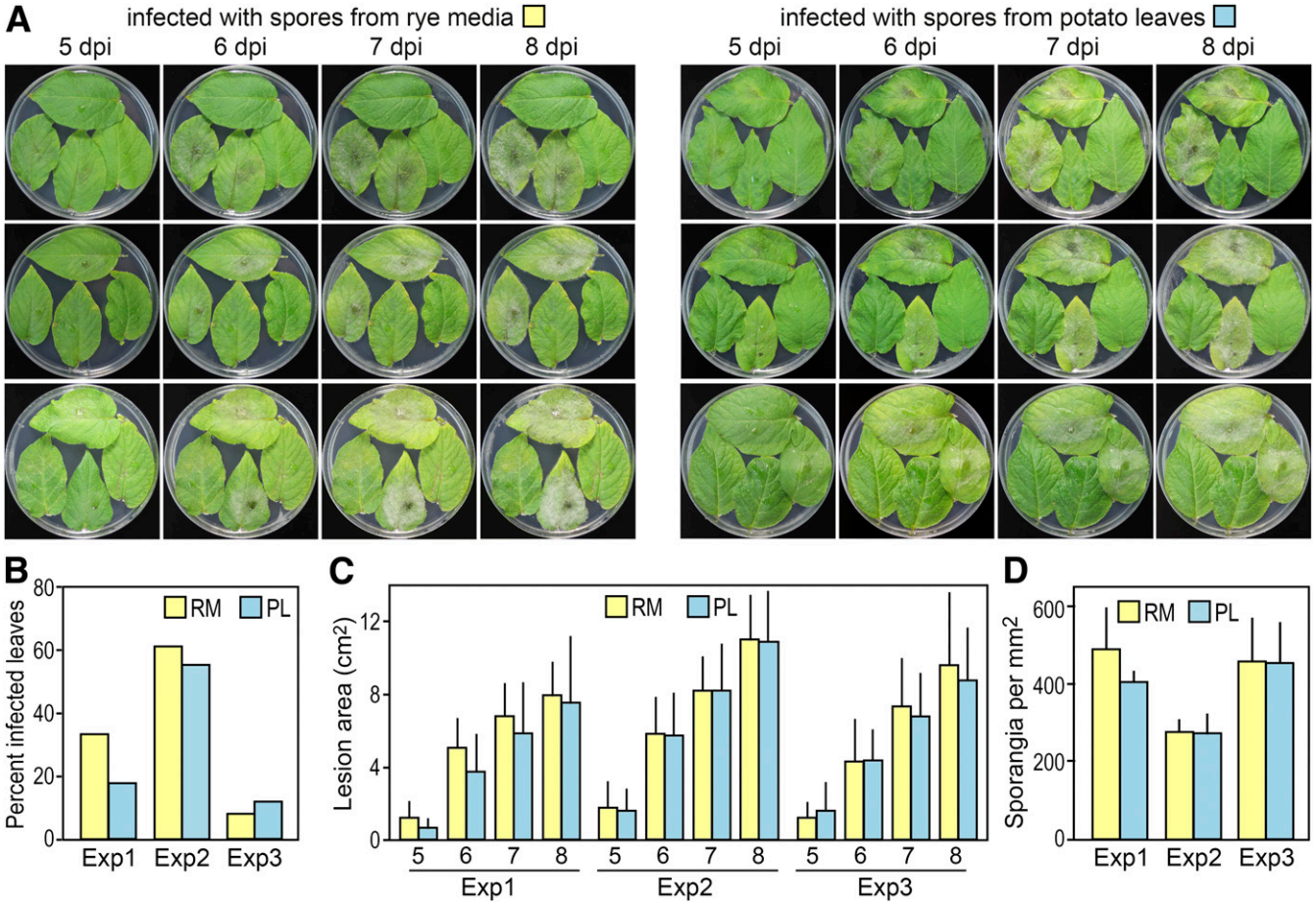

Fig. 6. Infection assays using zoospores from rye-sucrose media (RM) or potato leaflets (PL). **A,** Representative infected leaflets. Each row illustrates the same four leaflets between 5 and 8 days postinfection (dpi). In total, 100 leaflets were used per treatment per experiment. **B,** Percentage of leaflets showing infection in three independent experiments. **C,** Average size of lesions in each experiment. **D,** Sporangial density on infected leaflets.

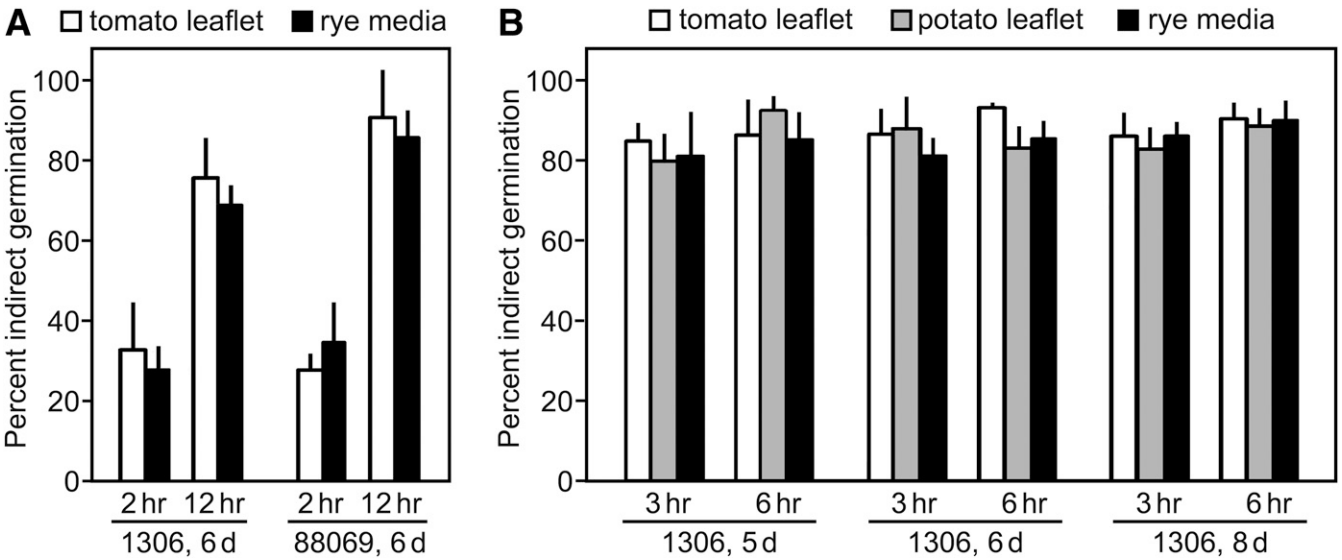

Fig. 7. Germination rates of sporangia. **A,** Indirect germination of isolates 1306 and 88069 measured 2 and 12 h after sporangia were cold treated. Sporangia were harvested from 6-day tomato leaflets or rye-sucrose cultures (inoculated by spreading with sporangia). Concentrations adjusted to 10^4 sporangia/ml prior to the cold treatment. **B,** Indirect germination of sporangia from isolate 1306 grown on tomato leaflets, potato leaflets, and spread-inoculated rye-sucrose media. Measurements were made 3 and 6 h after the cold treatment, using sporangia harvested 5, 6, and 8 days after inoculation.

rye-sucrose media and leaflets, respectively. Although the difference was not significant ($P = 0.13$), the trend toward higher germination in the plant-sourced material was interesting.

DISCUSSION

This study had two principal goals. One was to test whether sporangia from artificial media and plants were functionally equivalent. We conclude that the source of sporangia has a small effect on their transcriptomes but little or no impact on infection potential, germination, or pathogenic fitness. A second objective was to stress the importance of following "best practices" for handling sporangia. Not all experiments need to follow an exacting protocol but attention should be paid to the age of sporangia and how they treated during harvesting and germination. Marker genes that can indicate the purity, maturity, and germination status of sporangia were also described.

We are not the first to observe the finicky nature of *P. infestans* sporangia. For example, Sato (1994a) found that indirect germination varied depending on how long sporangia were allowed to mature after sporulation. After speculating that germination depended on the source of water in which sporangia were placed, he showed that pH and small differences in ion concentration influenced germination (Sato 1994b). This helps to explain why we believe that germination occurs more reliably in Petri's solution than water. Many laboratories germinate sporangia in deionized water (Boevink et al. 2016; Foolad et al. 2014; Fry et al. 2019) but this is an unnatural environment because leaf surfaces contain ions exuded from plant cells (Tukey 1970).

The extent of variation in the mRNA content of sporangia from artificial media compared with plant lesions was slight yet interesting, with an average of approximately 800 genes (4.4% of the total) exhibiting >twofold differences. We consider this to be a small number because, by comparison, 40 and 55% of genes were shown to vary by >twofold between mycelia and sporangia and between sporangia and chilled sporangia, respectively (Ah-Fong et al. 2017a). Because hyphae from plant tissues express more pathogenesis-promoting proteins such as RXLRs than do hyphae from artificial media, we had originally hypothesized that such factors would be inherited by plant-sourced sporangia and provide an advantage during early infection. However, this was not supported by our results. Nevertheless, we did see differences related to metabolism and possibly toxin efflux. Some of this could be attributed to the composition of the growth substrate such as the types and concentrations of cell wall glucans and soluble sugars. To understand such differences more fully, it would be necessary to study gene expression in hyphae, which was beyond the scope of this study.

As the writing of our manuscript was being completed, another laboratory reported using RNA-seq to compare *P. infestans* sporangia from plants and media (Fry et al. 2019). Sporangia from plug-inoculated rye-pea agar cultures after 12 days of growth were compared with sporangia from potato leaflets at 6 days postinfection. Approximately one-third of expressed genes (4,791 genes) were described as being >twofold differentially expressed between plant and media-sourced sporangia, which is 5.9 times more than in our analysis. Sporangia from plants were also reported to germinate >10-fold more than those from media, and to produce larger lesions on leaves. In contrast, our studies identified no differences in these characters. Several factors may explain the discrepancies. For example, the plant and media-sourced sporangia used in their RNA-seq study were very different in age, although we were careful to use sporangia of equal maturity. In addition, their germination assays were performed in water instead of Petri's solution, and different isolates, artificial media, and protocols for collecting sporangia were used. Our plant-sourced sporangia had also passed through plant tissue once, whereas they usually used two rounds of infection.

Although our study failed to repeat the findings of the other study, we do not dispute that passage through a host may be beneficial. Reports that the phenotypes of *Phytophthora* strains can change during culture go back at least 100 years (Rosenbaum 1917). The isolate used for most of our experiments, 1306, is diploid based on genome-wide counts of allele ratios (Matson 2018). However, many isolates of *P. infestans* exist in apparently unstable polyploid or aneuploid states which, in addition to point mutations, may cause variation in fitness (Li et al. 2017; Matson et al. 2015). Fitness may also change due to mitotic crossing-over, gene conversion, and epigenetic phenomena (Chamnanpunt et al. 2001; Lamour et al. 2012; Shrestha et al. 2016). Isolates may also acquire viruses which could affect phenotype (Cai et al. 2019). Passage through a plant may help eliminate some of these potentially deleterious features.

Despite the reputed variability of *P. infestans,* we do not believe that the species accumulates point mutations at unusually high rates. Based on the analysis of strains that have been serially propagated over five or more years and then subjected to Illumina sequencing, we calculate that approximately 10^{-8} changes occur per nucleotide per nuclear division. This is nearly identical to the mutation rates reported for human germline cells and *Arabidopsis thaliana*, which are approximately 1.4×10^{-8} and 7×10^{-7}, respectively (Acuna-Hidalgo et al. 2016; Watson et al. 2016). We calculate that two nucleotide changes may occur in genes during the growth of *P. infestans* across a 100-mm culture plate.

In summary, we agree with other researchers that changes in fitness (pathogenic or otherwise) can occur during the long-term cultivation of a pathogen. This phenomenon is well described in *P. infestans* (Hodgson and Sharma 1967) and is not unique to oomycetes or phytopathogens (Maury et al. 2017). Nevertheless, because we did not detect evidence of significant short-term effects resulting from cultivation in rye-sucrose media, studies of sporangia and other preinfection stages from media should yield results that are representative of the natural situation. This assumes that the delicate spore life stages are handled with appropriate care to avoid experimental artifacts. A more important question is whether results from one isolate of *P. infestans* can be extrapolated to others because much intraspecies diversity exists in traits relevant to disease such as host species preference, optimal temperature for spore germination, chemical sensitivity, and sexuality (Danies et al. 2013; Matson et al. 2015; Zhu et al. 2016). Isolate-specific transposons or chromosomal abnormalities may also increase the background mutation rate.

MATERIALS AND METHODS

Strains and culture conditions.

P. infestans strain 1306 was isolated from tomato in San Diego County, CA. Subsequent laboratory tests indicated that it is equally pathogenic to potato. The isolate was stored in liquid nitrogen, thawed, and then transferred approximately 10 times before being used for the experiments in this article. Isolate 88069 was isolated from potato in the Netherlands and provided by F. Govers. For routine cultures, plates were inoculated with a plug from an older plate and incubated at 18°C on rye-sucrose agar (rye A) in the dark (Caten and Jinks 1968). For the RNA-seq and sporulation time-course experiments, the plates were subjected to a cycle of 12 h of light and 12 h of darkness along with the plant material. For sporulation measurements on plates, the cultures were either plug inoculated or spread with

sporangia. The latter involved distributing 10^3 to 10^4 sporangia (in modified Petri's solution) per 100-mm plate with a bent glass rod. Plates were sealed in plastic boxes, which maintained relative humidity above 95% based on digital hygrometers. For some experiments described in the text, plates were viewed with a dissecting microscope to learn when sporangia began.

Sporangia were harvested from culture plates by pouring modified Petri's solution on top of each plate (10 ml for a 100-mm plate), followed by rubbing with a bent glass rod. Hyphal fragments were removed by filtration through 50-µm nylon mesh. Concentrations of sporangia were determined using a hemocytometer. If sporangia needed to be concentrated or pelleted, they were centrifuged at 700 × *g* in a swinging-bucket rotor for 4 min.

Zoosporogenesis was induced by placing sporangia at 10^4/ml in modified Petri's solution at 10°C. This involved filling a plastic tub with ice, placing a 6.3 mm thick sheet of acrylic on top of the ice, and then inverting a second tub on top. Temperature throughout the chamber was monitored using thermometers, which indicated that the air above the acrylic sheet became equilibrated to 10°C. Dishes containing sporangia were then placed on the acrylic sheet. Aliquots were removed at the times noted in Results and viewed under a microscope to assess indirect germination rates, basing measurements on a minimum of 100 sporangia.

Plant colonization.

Infections were performed using leaflets of tomato cultivar Pieralbo, leaflets of potato cultivar Russet Burbank, or russet tubers purchased locally. Plant materials were surface sterilized using dilute bleach for 5 min, washed twice in sterile water, blotted dry, and stored for 1 h before use. Leaflets were placed on 0.6% water agar plates in a sealed clear box with moist paper towels. Tuber slices were placed on a metal rack 8 mm above moist paper towels in the same type of box. This maintained the relative humidity between 95 and 98% based on a digital hygrometer. For general infections of leaflets and tubers, each was inoculated on their top surfaces with five drops of 15 µl of sporangia (2.5×10^4 sporangia/ml). Inoculated tubers were incubated in the dark at 18°C. Leaflets were incubated at 18°C with a cycle of 12 h of darkness and 12 h of light, using cool-white fluorescent lamps which provided an intensity of 95 µmoles/m^2/s at the leaf surface.

For assessing the infection potential of zoospores from different sources, the adaxial side of each leaflet was inoculated with a single zoospore in 10 µl of modified Petri's solution and incubated in the same way. Zoospores for this application were purified away from sporangia using a 15-µm nylon mesh filter. After inoculation, the infected materials were positioned in the incubator using a randomized complete block design and rotated daily. Starting on day 5, photographs were taken daily and later used to measure lesion sizes using Adobe Photoshop. At the end of the experiment, sporangia were washed from the leaflets and counted.

RNA analysis.

For RNA-seq analysis, sporangia were produced from rye-sucrose agar, infected leaflets, or infected tubers inoculated as described above. The sporangia were then harvested in modified Petri's solution by 30 s of gentle rubbing with a glass rod (agar plates) or vortexing (plant material). The liquid was then passed through a 50-µm nylon mesh, and the sporangia were pelleted at 700 × *g* for 4 min at room temperature. Care was taken to freeze the sporangia in liquid nitrogen within 10 min of adding Petri's solution. After grinding the tissues under liquid nitrogen, RNA was isolated using the Spectrum Plant Total RNA kit from Sigma.

Indexed libraries for RNA-seq were prepared using the Illumina TruSeq kit and sequenced using an Illumina NextSeq 500, obtaining single-end 75-nt reads. These were filtered and mapped to the *P. infestans* genome using Bowtie 2.2.5 and Tophat 2.0.14, allowing for one mismatch per read (Langmead et al. 2009). Expression and differential expression calls were made with edgeR using TMM normalization, a generalized linear model, and FDR calculations based on the Benjamini-Hochberg method (Robinson et al. 2010). One of the three biological replicates from the harvest 2 tubers was omitted from this analysis because it appeared to be an outlier, apparently due to bacterial growth on the tuber. Hierarchical clustering, heatmap generation, and PCA were performed using Partek Genomics Suite. GO term enrichment analysis was performed using the GOHyperGAll script (Horan et al. 2008). Analyses of metabolism used functional assignments obtained using a reannotation of the *P. infestans* genome (Kagda 2017), in which enzymes were assigned to pathways based on the KEGG classification scheme (Aoki-Kinoshita and Kanehisa 2007). Expression comparisons entailed adding together the FPKM values of genes with the same function (generally by Enzyme Commission number), and then aggregating the FPKM of all genes encoding all enzymes in each KEGG pathway.

Microarray studies were performed on custom Affymetrix arrays. These were prepared and hybridized with RNA probes, as described (Randall et al. 2005). RNA samples came from sporangia that were harvested from strain 88069 and chilled at 4°C for the times indicated in Results.

Calculation of mutation rates.

After single-nuclear purification through the zoospore stage, independent lineages of isolate 1306 were established and grown for 20 serial transfers on 60-mm cultures of rye-sucrose agar. DNA extracted from the early and terminal stages of each lineage was then used for sequencing, obtaining 50-nt paired-end data to approximately 60-fold coverage using an Illumina HiSeq 2500. The data were then analyzed using the Var-Seq pipeline implemented in systemPipeR (Backman and Girke 2016). In brief, this involved filtering and alignment to the reference genome using BWA, and the use of GATK (McKenna et al. 2010) to identify sequence polymorphisms. Because *P. infestans* is diploid, only cases where the sequences of the lineages were entirely different or a homozygote changed to a heterozygote (or vice versa) could be scored. We estimate that each lineage had undergone between 162 and 332 nuclear divisions, based on a measurement of the increase in nuclei during growth across a 60-mm culture plate and the doubling time of nuclei determined in a previous study (Ah Fong and Judelson 2003). In comparisons of three lineages, on average, 1,015 single-nucleotide variants were identified per lineage, which corresponds to approximately 10^{-8} changes per nucleotide per nuclear division. This may be an underestimate because some deleterious mutations would be lost during propagation.

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