Phytophthora infestans sporangia produced in artificial media and plant lesions have subtly divergent transcription profiles but equivalent infection potential and aggressiveness

Wiphawee Leesutthiphonchai and Howard S. Judelson*

Department of Microbiology and Plant Pathology

University of California

Riverside, CA 92521 USA

* For correspondence: howard.judelson@ucr.edu; Tel 951-827-4199

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

ABSTRACT

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 since 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.

INTRODUCTION

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-Melendez 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 to 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. 2018).

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. While 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 about 5 days, which is recorded as Day 0 in Fig. 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. As shown by the dashed line in Fig. 1A, cultures grown in this matter accrue sporangia over at least six days. Many papers describe collecting sporangia after 10 to 14 days; the sporangia would thus range from about 1 to 10 days

in age.

That age is an important variable is illustrated in Fig. 1B, which shows the relative fraction of sporangia releasing zoospores (*i.e.* indirect germination) in isolates 1306 and 88069 after 3 hr 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. 1967; 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 release decreases in cultures that have aged over a time period similar to that shown in Fig. 1B (King et al. 1968).

We play 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, 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, since 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 since this transcriptional inhibitor slowly crosses the sporangial wall (Judelson and Roberts 2002). Instead, we rush to harvest each batch of sporangia within 8 to 10 min. For RNA or protein analysis, this means 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. 2018), but we use the minimum possible (700 $\times g$) since 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 Fig. 1A in order to represent sporangia on the first and second major days of sporulation. This corresponded to 4.5 and 5.5 days post-infection 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-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 both to the source of sporangia and the harvest date. This and subsequent analyses were limited to the 15,522 genes having 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 >2-fold differences based on a Benjamini-Hochberg false discovery rate (FDR) threshold of 0.05. This variation is evident in the heatmap in Fig. 2A and enumerated in Fig. 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.

While 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 harvest 2 sporangia from rye media, an average of 316 genes exhibited >2-fold 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 since 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 to 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 Fig. 3B show a similar experiment using RNA-seq, where chilling increased the FPKM value (fragments per kilobase of exon per million mapped reads) by at least 100-fold. The other lanes represent the 24 sporangia samples from rye media, potato and tomato leaflets, and potato tubers. None expresses 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 Fig. 2 and our supplemental tables.

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 mis-timed the two harvests. One gene used for this analysis was PITG_21410, which encodes elicitin-like protein INF4 (Jiang et al. 2005). As shown on the left side of Fig. 3C, the mRNA level of this gene declines in sporulating cultures and is very low in sporangia. As shown on the right side

of Fig. 3C, all 24 rye media, leaflet, and tuber samples exhibited very low levels of expression of this gene. 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 Fig. 2 and our supplemental 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 examples, over-represented GO terms included oxidoreductase activity (GO:0016491, *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 since only the leaflets contain appreciable amounts of cutin. Also logical is our observation that sporangia from rye media contained higher levels of xylosidase mRNA, since 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 about 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 about 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 to rye-sucrose media. Such proteins are thought to participate in the efflux of toxins including plant defense molecules (Perlin et al. 2014). As shown in Fig. 4C, the ratio of mRNA abundance in sporangia harvested from tomato leaflets versus rye-sucrose media was skewed towards higher expression in the leaflets, 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 to 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

247 all samples.

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

257 Expressed at CPM>1 in sporangia were 114 CRN genes. However, none of their
258 mRNA levels were consistently higher in sporangia from a plant source compared to
259 rye-sucrose media, although PITG_19565 was 2-fold higher in potato leaflets compared
260 to media. Instead, CRN genes were more likely to exhibit higher mRNA levels in
261 sporangia from media.

262 Transcript levels of genes associated with sporulation varied little between sporangia
263 sourced from plants and media. This conclusion is based on the analysis of genes that
264 encode proteins specific to the flagellar axoneme (Judelson et al. 2012). This is
265 expected since inhibitor studies demonstrated that most zoospore proteins are
266 preformed in sporangia and not translated during germination (Clark et al. 1978). Due to
267 normal mRNA decay in aging sporangia, it was also not surprising that the transcript
268 levels of most flagella-associated genes were lower in harvest 2 versus the harvest 1
269 sporangia, as 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% 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 six days after infection than from artificial media cultures 12 days after inoculation (Fry et al. 2018). Although that study 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 to 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 to 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 hr, zoospores were separated from sporangia by filtration through 15 µm mesh, diluted to 100 per ml, and individual leaflets inoculated with a single drop bearing one zoospore. This was repeated in three separate experiments, using 100 leaflets per treatment per experiment, which after inoculation 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, B). There was no significant difference based on one-way ANOVA and Wilcoxon signed rank tests. The average infection potential of each zoospore was 32%, which is in the range described for *Phytophthora 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 since 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 post-infection in the three single-zoospore experiments (Fig. 6C). Moreover, the total amount of

316 sporangia produced on the leaflets by day 8 was similar regardless of whether the
317 inoculum was from leaflets or artificial media (Fig. 6D). Therefore, similar
318 aggressiveness was exhibited by sporangia from both sources.
319 Since foliage infections are usually initiated by sporangia, we also attempted to
320 assess whether sporangia from plants and rye-sucrose media had similar infection
321 potentials. This involved placing a droplet of water containing a single sporangium on
322 each of multiple leaflets, which were placed at 10°C for 4 hr to trigger zoosporogenesis
323 and then incubated at 18°C in a plant growth chamber. Excluding cases where the
324 inoculum drop appeared to have run off the leaflet, close to 100% of leaflets became
325 infected. Since the rate of colonization was so high, we cannot exclude the possibility
326 that miniscule differences may exist in the infection potential of sporangia from plants
327 and media, but such small differences may not be biologically relevant.
328
329 **Sporangia from plants and artificial media germinate at similar rates**
330 Although the rates of zoosporogenesis of the two types of sporangia were not
331 quantified precisely in the plant infection experiments described above, they appeared
332 similar based on microscopic examination. Several additional studies confirmed that
333 zoosporogenesis progresses similarly in plant and media-sourced sporangia.
334 In the first experiment, we quantified zoospore release from isolates 1306 and
335 88069. This used sporangia that had been harvested from tomato leaflets and media six
336 days after inoculation. With both strains, the source of sporangia had little if any effect
337 on germination based on observations made 2 and 12 hr after chilling the spores (Fig.
338 7A). For example, after 12 hr 76±10% and 69±5% of sporangia from isolate 1306

339 sourced from tomato leaflets and rye-sucrose media had germinated, respectively.
340 Since zoospores from plant and media were shown above to have similar infection
341 potentials, it follows that the same should hold for sporangia.
342 A second study also compared the germination of sporangia obtained from tomato
343 leaflets, potato leaflets, and media, this time using only isolate 1306 (Fig. 7B). This
344 experiment also evaluated sporangia harvested 5, 6, and 8 days after inoculation.
345 Based on observations made 3 hr and 6 hr after chilling, the source of sporangia had
346 little effect on germination. This provides further support to the hypothesis that growing
347 *P. infestans* on a plant does confer upon its sporangia a higher infection potential. It is
348 interesting that the 5-day and 8-day sporangia germinated at similar rates in this study,
349 while germination was impaired in the older cultures in the Fig. 1B experiment. We
350 believe that this is because the cultures in Fig. 1B were plug-inoculated, while those in
351 Fig. 7B were initiated by spreading sporangia. Since hyphal growth is denser in plug-
352 inoculated cultures, sporangia germination may be influenced by concentrations of
353 nutrients or waste products in the underlying media. This observation stresses the
354 importance of preparing sporangia under standardized conditions.
355 We also measured the germination of cysts derived from the plant and media-
356 sourced zoospores used in the infection assays shown in Fig. 6. This involved placing
357 aliquots of the zoospores in clarified rye-sucrose broth to trigger encystment and
358 germination. The cyst germination rates in the three experiments in Fig. 6 averaged 69
359 and 78% for sporangia from rye-sucrose media and leaflets, respectively. While the
360 difference was not significant (P=0.13), the trend towards higher germination in the
361 plant-sourced material was interesting.

DISCUSSION

This study had two principal goals. One was to test if 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 found that indirect germination varied depending on how long sporangia were allowed to mature after sporulation (Sato 1994a). 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. 2018), but this is an unnatural environmental since leaf surfaces contain ions exuded from plant cells (Tukey 1970).

The extent of variation in the mRNA content of sporangia from artificial media compared to plant lesions was slight yet interesting, with about an average of about 800 genes (4.4% of the total) exhibiting >2-fold differences. We consider this to be a small number since by comparison 40% and 55% of genes were shown to vary by >2-fold

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. 2018). Sporangia from plug-inoculated rye-pea agar cultures after 12 days of growth were compared with sporangia from potato leaflets at 6 days post-infection. About one-third of expressed genes (4,791) were described as being >2-fold 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, while 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, while they usually used two rounds of infection.

While 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. 2018). 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 about 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 about 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, since we did not detect evidence of significant short-term effects resulting from cultivation in rye-sucrose media, studies of sporangia and other pre-infection stages from media should yield results that are representative of the natural situation. This assumes that these 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 since 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 ten times before being used for the experiments in this paper. 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 timecourse experiments, the plates were subjected to a 12 hr light/dark cycle 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 $\times g$ 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 1/4-inch 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.

477 **Plant colonization**

478 Infections were performed using leaflets of tomato cultivar Pieralbo, leaflets of potato
479 cultivar Russet Burbank, or russet tubers purchased locally. Plant materials were
480 surface-sterilized using dilute bleach for 5 min, washed twice in sterile water, blotted
481 dry, and stored for 1 hr before use. Leaflets were placed on 0.6% water agar plates in a
482 sealed clear box with moist paper towels. Tuber slices were placed on a metal rack 8-
483 mm above moist paper towels in the same type of box. This maintained the relative
484 humidity between 95 and 98% based on a digital hygrometer. For general infections of
485 leaflets and tubers, each was inoculated on their top surfaces with five drops of 15 µl
486 sporangia (2.5 x 10^4 per ml). Inoculated tubers were incubated in the dark at 18°C.
487 Leaflets were incubated at 18°C with a 12 hr dark-light cycle, using cool-white
488 fluorescent lamps which provided an intensity of 95 µmoles/m^2/s at the leaf surface.
489 For assessing the infection potential of zoospores from different sources, the adaxial
490 side of each leaflet was inoculated with a single zoospore in 10 µl of modified Petri's
491 solution and incubated in the same way. Zoospores for this application were purified
492 away from sporangia using a 15 µm nylon mesh filter. After inoculation, the infected
493 materials were positioned in the incubator using a randomized complete block design
494 and rotated daily. Starting on day 5, photographs were taken daily and later used to
495 measure lesion sizes using Adobe Photoshop. At the end of the experiment, sporangia
496 were washed from the leaflets and counted.
497
498 **RNA analysis**
499 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 sec 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 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 since it appeared to be an outlier, apparently due to bacterial growth on the tuber. Hierarchical clustering, heatmap generation, and PCA analysis 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 about 60-fold coverage using an Illumina HiSeq 2500. The data was 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. Since *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, an average of 1015 single-nucleotide variants were identified per lineage, which corresponds to about 10^{-8} changes per nucleotide per nuclear division. This may be an underestimate since some deleterious mutations would

546 be lost during propagation.

547

548 **ACKNOWLEDGEMENTS**

549 This work was supported by grants to HSJ from the United States Department of
550 Agriculture, National Institute of Food and Agriculture and the National Science
551 Foundation. WL was supported by a Royal Thai Government scholarship. We thank
552 Andrea Vu for sharing data.

553

554 **REFERENCES**

555 Abrahamian, M., Ah-Fong, A. M., Davis, C., Andreeva, K., and Judelson, H. S. 2016.
556 Gene expression and silencing studies in *Phytophthora infestans* reveal
557 infection-specific nutrient transporters and a role for the nitrate reductase
558 pathway in plant pathogenesis. PLoS Pathog. 12:e1006097.
559 Acuna-Hidalgo, R., Veltman, J. A., and Hoischen, A. 2016. New insights into the
560 generation and role of *de novo* mutations in health and disease. Genome Biol
561 17:241.
562 Ah Fong, A. M., and Judelson, H. S. 2003. Cell cycle regulator cdc14 is expressed
563 during sporulation but not hyphal growth in the fungus-like oomycete
564 *Phytophthora infestans.* Mol. Microbiol. 50:487-494.
565 Ah-Fong, A. M., Kim, K. S., and Judelson, H. S. 2017a. Rna-seq of life stages of the
566 oomycete *Phytophthora infestans* reveals dynamic changes in metabolic, signal
567 transduction, and pathogenesis genes and a major role for calcium signaling in
568 development. BMC Genomics 18:198.

569 Ah-Fong, A. M., Shrivastava, J., and Judelson, H. S. 2017b. Lifestyle, gene gain and
570 loss, and transcriptional remodeling cause divergence in the transcriptomes of
571 *Phytophthora infestans* and *Pythium ultimum* during potato tuber colonization.
572 BMC Genomics 18:e764.
573 Aoki-Kinoshita, K. F., and Kanehisa, M. 2007. Gene annotation and pathway mapping in
574 kegg. Methods Mol. Biol. 396:71-91.
575 Backman, T. W. H., and Girke, T. 2016. Systempiper: NGS workflow and report
576 generation environment. BMC Bioinformatics 17.
577 Belhaj, K., Cano, L. M., Prince, D. C., Kemen, A., Yoshida, K., Dagdas, Y. F.,
578 Etherington, G. J., Schoonbeek, H. J., van Esse, H. P., Jones, J. D. G., Kamoun,
579 S., and Schornack, S. 2017. *Arabidopsis* late blight: Infection of a nonhost plant
580 by *Albugo laibachii* enables full colonization by *Phytophthora infestans*. Cell.
581 Microbiol. 19:e12628.
582 Blaschek, W., Haass, D., Koehler, H., and Franz, G. 1981. Cell-wall regeneration by
583 *Nicotiana tabacum* protoplasts - chemical and biochemical aspects. Plant Sci.
584 Lett. 22:47-57.
585 Boevink, P. C., Wang, X. D., McLellan, H., He, Q., Naqvi, S., Armstrong, M. R., Zhang,
586 W., Hein, I., Gilroy, E. M., Tian, Z. D., and Birch, P. R. J. 2016. A *Phytophthora*
587 *infestans* RXLR effector targets plant PP1c isoforms that promote late blight
588 disease. Nat. Comm. 7:10311.
589 Bradshaw, J. E., Bryan, G. J., Lees, A. K., McLean, K., and Solomon-Blackburn, R. M.
590 2006. Mapping the R10 and R11 genes for resistance to late blight *(phytophthora*

591 *infestans*) present in the potato *(Solanum tuberosum)* R-gene differentials of
592 black. Theor. Appl. Genet. 112:744-751.
593 Brasier, C. M., and Griffin, M. J. 1979. Taxonomy of *Phytophthora palmivora* on cocoa.
594 T. Brit. Mycol. Soc 72:111-143.
595 Byrt, P., and Grant, B. R. 1979. Some conditions governing zoospore production in
596 axenic cultures of *Phytophthora cinnamomi* rands. Aust. J. Bot. 27:103-115.
597 Cai, G., Myers, K., Fry, W. E., and Hillman, B. I. 2018. *Phytophthora infestans* RNA
598 virus 2, a novel RNA virus from *Phytophthora infestans,* does not belong to any
599 known virus group. Arch. Virol. in press.
600 Caten, C. E., and Jinks, J. L. 1968. Spontaneous variability in isolates of *Phytophthora*
601 *infestans.* I. Cultural variation. Can. J. Bot 46:329-348.
602 Chamnanpunt, J., Shan, W.-X., and Tyler, B. M. 2001. High frequency mitotic gene
603 conversion in genetic hybrids of the oomycete *Phytophthora sojae*. Proc. Natl.
604 Acad. Sci. USA 98:14530-14535.
605 Clark, M. C., Melanson, D. L., and Page, O. T. 1978. Purine metabolism and differential
606 inhibition of spore germination in *Phytophthora infestans*. Can. J. Microbiol.
607 24:1032-1038.
608 Danies, G., Small, I. M., Myers, K., Childers, R., and Fry, W. E. 2013. Phenotypic
609 characterization of recent clonal lineages of *Phytophthora infestans* in the united
610 states. Plant Dis. 97:873-881.
611 Elsner, P., VanderMolen, G., Horton, J. C., and Bowen, C. C. 1967. Fine structure of
612 sporangia of *Phytophthora infestans* during temperature-induced differentation.
613 Phytopathology 54:1765-1177.

614 Foolad, M. R., Sullenberger, M. T., Ohlson, E. W., and Gugino, B. K. 2014. Response of
615 accessions within tomato wild species, *Solanum pimpinellifolium* to late blight.
616 Plant Breeding 133:401-411.
617 Fry, W., Patev, S. P., Meyers, K., Bao, K., and Fei, Z. 2018. *Phytophthora infestans*
618 sporangia produced in culture and on tomato leaflet lesions show marked
619 differences in indirect germination rates, aggressiveness, and global transcription
620 profiles. Mol Plant Microbe Interact doi:10.1094/MPMI-09-18-0255-TA.
621 Fry, W. E. 2016. *Phytophthora infestans:* New tools (and old ones) lead to new
622 understanding and precision management. Annu. Rev. Phytopathol. 54:529-547.
623 Gamboa-Melendez, H., Huerta, A. I., and Judelson, H. S. 2013. BZIP transcription
624 factors in the oomycete *Phytophthora infestans* with novel DNA-binding domains
625 are involved in defense against oxidative stress. Eukaryot. Cell 12:1403-1412.
626 Haas, B. J., Kamoun, S., Zody, M. C., Jiang, R. H., Handsaker, R. E., Cano, L. M.,
627 Grabherr, M., Kodira, C. D., Raffaele, S., Torto-Alalibo, T., Bozkurt, T. O., Ah-
628 Fong, A. M., Alvarado, L., Anderson, V. L., Armstrong, M. R., Avrova, A., Baxter,
629 L., Beynon, J., Boevink, P. C., Bollmann, S. R., Bos, J. I., Bulone, V., Cai, G.,
630 Cakir, C., Carrington, J. C., Chawner, M., Conti, L., Costanzo, S., Ewan, R.,
631 Fahlgren, N., Fischbach, M. A., Fugelstad, J., Gilroy, E. M., Gnerre, S., Green, P.
632 J., Grenville-Briggs, L. J., Griffith, J., Grunwald, N. J., Horn, K., Horner, N. R., Hu,
633 C. H., Huitema, E., Jeong, D. H., Jones, A. M., Jones, J. D., Jones, R. W.,
634 Karlsson, E. K., Kunjeti, S. G., Lamour, K., Liu, Z., Ma, L., Maclean, D.,
635 Chibucos, M. C., McDonald, H., McWalters, J., Meijer, H. J., Morgan, W., Morris,
636 P. F., Munro, C. A., O'Neill, K., Ospina-Giraldo, M., Pinzon, A., Pritchard, L.,

637 Ramsahoye, B., Ren, Q., Restrepo, S., Roy, S., Sadanandom, A., Savidor, A.,
638 Schornack, S., Schwartz, D. C., Schumann, U. D., Schwessinger, B., Seyer, L.,
639 Sharpe, T., Silvar, C., Song, J., Studholme, D. J., Sykes, S., Thines, M., van de
640 Vondervoort, P. J., Phuntumart, V., Wawra, S., Weide, R., Win, J., Young, C.,
641 Zhou, S., Fry, W., Meyers, B. C., van West, P., Ristaino, J., Govers, F., Birch, P.
642 R., Whisson, S. C., Judelson, H. S., and Nusbaum, C. 2009. Genome sequence
643 and analysis of the Irish potato famine pathogen *Phytophthora infestans*. Nature
644 461:393-398.
645 Halsall, D. M., and Forrester, R. I. 1977. Effects of certain cations on formation and
646 infectivity of *Phytophthora* zoospores.1. Effects of calcium, magnesium,
647 potassium, and iron ions. Can. J. Microbiol. 23:994-1001.
648 Hardham, A. R., and Hyde, G. J. 1997. Asexual sporulation in the oomycetes. Adv.
649 Botan. Res. 24:353-398.
650 Hodgson, W. A., and Sharma, K. P. 1967. Restoration of virulence of stored cultures of
651 *Phytophthora infestans.* Can. J. Plant Sci. 47:447-8.
652 Horan, K., Jang, C., Bailey-Serres, J., Mittler, R., Shelton, C., Harper, J. F., Zhu, J. K.,
653 Cushman, J. C., Gollery, M., and Girke, T. 2008. Annotating genes of known and
654 unknown function by large-scale coexpression analysis. Plant Physiol. 147:41-
655 57.
656 Jarvis, M. C., Hall, M. A., Threlfall, D. R., and Friend, J. 1981. The polysaccharide
657 structure of potato cell walls - chemical fractionation. Planta 152:93-100.

658 Jiang, R. H., Tyler, B. M., Whisson, S. C., Hardham, A. R., and Govers, F. 2005.
659 Ancient origin of elicitin gene clusters in *Phytophthora* genomes. Mol. Biol. Evol.
660 23:338-351.
661 Judelson, H. S., and Roberts, S. 2002. Novel protein kinase induced during sporangial
662 cleavage in the oomycete *Phytophthora infestans*. Eukaryot. Cell 1:687-695.
663 Judelson, H. S., Narayan, R. D., and Tani, S. 2009. Metabolic adaptation of
664 *phytophthora infestans* during growth on leaves, tubers, and artificial media. Mol.
665 Plant Pathol. 10:843-855.
666 Judelson, H. S., Shrivastava, J., and Manson, J. 2012. Decay of genes encoding the
667 oomycete flagellar proteome in the downy mildew *Hyaloperonospora*
668 *arabidopsidis*. PLOS One 7:e47624.
669 Kagda, M. 2017. Insight into the dynamic metabolism of *Phytophthora infestans*, Ph.D.
670 thesis, University of California, Riverside.
671 King, J. E., Colhoun, J., and Butler, R. D. 1968. Changes in the ultrastructure of
672 sporangia of *Phytophthora infestans* associated with indirect germination and
673 aging. Trans Brit Mycol Soc 154:1794-1804.
674 Kirk, W. W., Abu-El Samen, F. M., Muhinyuza, J. B., Hammerschmidt, R., Douches, D.
675 S., Thill, C. A., Groza, H., and Thompson, A. L. 2005. Evaluation of potato late
676 blight management utilizing host plant resistance and reduced rates and
677 frequencies of fungicide applications. Crop Protect. 24:961-970.
678 Knudsen, K. E. B. 2014. Fiber and nonstarch polysaccharide content and variation in
679 common crops used in broiler diets. Poultry Sci. 93:2380-2393.

680 Kong, P., and Hong, C. 2016. Soil bacteria as sources of virulence signal providers
681 promoting plant infection by *Phytophthora* pathogens. Sci. Rep. 6:33239.
682 Lamour, K., Mudge, J., Gobena, D., Hurtado-Gonzales, O. P., Schmutz, J., Kuo, A.,
683 Miller, N. A., Rice, B. J., Raffaele, S., Cano, L., Bharti, A. K., Donahoo, R. S.,
684 Finley, S. L., Huitema, E., Hulvey, J., Platt, D., Salamov, A., Savidor, A., Sharma,
685 R., Stam, R., Storey, D., Thines, M., Win, J., Haas, B., Dinwiddie, D., Jenkins, J.,
686 Knight, J., Affourtit, J., Han, C. S., Chertkov, O., Lindquist, E. A., Detter, C.,
687 Grigoriev, I. V., Kamoun, S., and Kingsmore, S. F. 2012. Genome sequencing
688 and mapping reveal loss of heterozygosity as a mechanism for rapid adaptation
689 in the vegetable pathogen *Phytophthora capsici.* Mol. Plant Microbe Interact.
690 25:1350-1360.
691 Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. 2009. Ultrafast and memory-
692 efficient alignment of short DNA sequences to the human genome. Genome Biol.
693 10:R25.
694 Latijnhouwers, M., Ligterink, W., Vleeshouwers, V. G. A. A., Van West, P., and Govers,
695 F. 2004. A G-alpha subunit controls zoospore motility and virulence in the potato
696 late blight pathogen *Phytophthora infestans*. Molec. Microbiol. 51:925-936.
697 Leesutthiphonchai, W., and Judelson, H. S. 2018. A MADS-box transcription factor
698 regulates a central step in sporulation of the oomycete *Phytophthora infestans*.
699 Mol. Microbiol. 110:562-575.
700 Leesutthiphonchai, W., Vu, A. L., Ah-Fong, A. M. V., and Judelson, H. S. 2018. How
701 does *Phytophthora infestans* evade control efforts? Modern insight into the late
702 blight disease. Phytopathology 108:916-924.

703 Lew, R. R. 2011. How does a hypha grow? The biophysics of pressurized growth in
704 fungi. Nat. Rev. Microbiol. 9:509-518.
705 Li, Y., Shen, H., Zhou, Q., Qian, K., van der Lee, T., and Huang, S. W. 2017. Changing
706 ploidy as a strategy: The Irish potato famine pathogen shifts ploidy in relation to
707 its sexuality. Mol. Plant Microbe Interact. 30:45-52.
708 Matson, M. E. H. 2018. A chromosome-scale reference assembly facilitates new
709 insights into fungicide sensitivity and genome plasticity in *Phytophthora infestans*,
710 Ph.D. thesis, University of California, Riverside.
711 Matson, M. E. H., Small, I. M., Fry, W. E., and Judelson, H. S. 2015. Metalaxyl
712 resistance in *Pytophthora infestans:* Assessing role of *Rpa190* gene and diversity
713 within clonal lineages. Phytopathology 105:1594-1600.
714 Maury, M. M., Chenal-Francisque, V., Bracq-Dieye, H., Han, L., Leclercq, A., Vales, G.,
715 Moura, A., Gouin, E., Scortti, M., Disson, O., Vazquez-Boland, J. A., and Lecuit,
716 M. 2017. Spontaneous loss of virulence in natural populations of *Listeria*
717 *monocytogenes.* Infect. Immun. 85:e00541-17
718 McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A.,
719 Garimella, K., Altshuler, D., Gabriel, S., Daly, M., and DePristo, M. A. 2010. The
720 genome analysis toolkit: A MapReduce framework for analyzing next-generation
721 DNA sequencing data. Genome Res. 20:1297-1303.
722 Mizubuti, E. S. G., Aylor, D. E., and Fry, W. E. 2000. Survival of *Phytophthora infestans*
723 sporangia exposed to solar radiation. Phytopathology 90:78-84.

724 Naess, S. K., Bradeen, J. M., Wielgus, S. M., Haberlach, G. T., McGrath, J. M., and
725 Helgeson, J. P. 2000. Resistance to late blight in *Solanum bulbocastanum* is
726 mapped to chromosome 8. Theor. Appl. Genet. 101:697-704.
727 Perlin, M. H., Andrews, J., and Toh, S. S. 2014. Essential letters in the fungal alphabet:
728 ABC and MFS transporters and their roles in survival and pathogenicity. Adv.
729 Genet. 85:201-253.
730 Petri, L. 1917. Research on morphology and biology of *Blepharospora cambivora*
731 *parasitica* from chestnut (in Italian). Atti R. Accad. Lincei 26:297-299.
732 Randall, T. A., Dwyer, R. A., Huitema, E., Beyer, K., Cvitanich, C., Kelkar, H., Ah Fong,
733 A. M. V., Gates, K., Roberts, S., Yatzkan, E., Gaffney, T., Law, M., Testa, A.,
734 Torto, T., Zhang, M., Zheng, L., Mueller, E., Windass, J., Binder, A., Birch, P. R.
735 J., Gisi, U., Govers, F., Gow, N. A. R., Mauch, F., van West, P., Waugh, M. E.,
736 Yu, J., Boller, T., Kamoun, S., Lam, S. T., and Judelson, H. S. 2005. Large-scale
737 gene discovery in the oomycete *Phytophthora infestans* reveals likely
738 components of phytopathogenicity shared with true fungi. Mol. Plant Microbe
739 Interact. 18:229-243.
740 Robinson, M. D., McCarthy, D. J., and Smyth, G. K. 2010. EdgeR: A bioconductor
741 package for differential expression analysis of digital gene expression data.
742 Bioinformatics 26:139-140.
743 Rosenbaum, J. 1917. Studies of the genus *Phytophthora*. Proc. Natl. Acad. Sci USA
744 3:159-163.
745 Sato, N. 1994a. Maturation of sporangia of *Phytophthora infestans* affecting the rapidity
746 of indirect germination. Ann. Phytopath. Soc. Japan 60:53-59.

747 Sato, N. 1994b. Effect of some inorganic salts and hydrogen ion concentration on
748 indirect germination of the sporangia of *Phytophthora infestans*. Ann. Phytopath.
749 Soc. Japan 60:441-447.
750 Shrestha, S. D., Chapman, P., Zhang, Y., and Gijzen, M. 2016. Strain specific factors
751 control effector gene silencing in *Phytophthora sojae*. PLOS One 11:e0150530.
752 Soto, T., Nunez, A., Madrid, M., Vicente, J., Gacto, M., and Cansado, J. 2007.
753 Transduction of centrifugation-induced gravity forces through mitogen-activated
754 protein kinase pathways in the fission yeast *Schizosaccharomyces pombe*.
755 Microbiology 153:1519-1529.
756 Tani, S., Yatzkan, E., and Judelson, H. S. 2004. Multiple pathways regulate the
757 induction of genes during zoosporogenesis in *Phytophthora infestans*. Mol. Plant
758 Microbe Interact. 17:330-337.
759 Tukey, H. B. J. 1970. The leaching of substances from plants. Annu. Rev. Plant Physiol.
760 Plant Mol. Biol. 21:305-324.
761 Vanbruggen, A. H. C., Osmeloski, J. F., and Jacobson, J. S. 1987. Effects of simulated
762 acidic mist on germination of *Alternaria solani* and *Phytophthora infestans* n vitro
763 and their infection efficiency and sporulation on potato. Phytopathology 77:564-
764 570.
765 Walker, C., and van West, P. 2007. Zoospore development in the oomycetes. Fungal
766 Biol. Rev. 21:10-18.
767 Watson, J. M., Platzer, A., Kazda, A., Akimcheva, S., Valuchova, S., Nizhynska, V.,
768 Nordborg, M., and Riha, K. 2016. Germline replications and somatic mutation

769 accumulation are independent of vegetative life span in *Arabidopsis*. Proc. Natl.
770 Acad. Sci. USA 113:12226-12231.
771 Zhu, W., Shen, L. L., Fang, Z. G., Yang, L. N., Zhang, J. F., Sun, D. L., and Zhan, J. S.
772 2016. Increased frequency of self-fertile isolates in *Phytophthora infestans* may
773 attribute to their higher fitness relative to the A1 isolates. Sci. Rep. 6: 29428
774
775
776

FIGURE LEGENDS

Fig. 1. Influence of age on sporulation and germination in *P. 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 percent of maximum, which equaled 710±69, 247±25, and 331±56 sporangia per 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 hr after being placed in Petri's solution at 10°C. The values are expressed relative to the maximum for each isolate, which equaled 82% and 71% germination for isolates 88069 and 1306 on day 4, respectively. 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.

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 times labeled "harvest 1" and "harvest 2" in Fig. 1A, 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.

800 Each symbol represents a biological replicate. **C,** Number of genes that were
801 differentially expressed between culture conditions, based on thresholds of >2-fold
802 change and FDR<0.05 at the two timepoints. **D,** Number of genes differentially
803 expressed between sporangia from the harvest 1 and harvest 2 timepoints (*i.e.* 24 hr
804 apart).
805
806 **Fig. 3.** Quality assessments of the 24 sporangia samples. **A,** Expression of
807 zoosporogenesis-induced gene PITG_13579 in sporangia immediately after harvesting
808 and after 10, 30, 50, and 70 min of chilling in modified Petri's solution. Data are based
809 on microarray analysis. **B,** Abundance of PITG_13579 mRNA based on RNA-seq. The
810 two left samples are freshly harvested sporangia and the same chilled for 0 and 60 min
811 (chilled spor.). Samples labeled RM, TL, PL, and PT are sporangia from rye media,
812 tomato leaflets, potato leaflets, and potato tubers, respectively, from harvest 1 and
813 harvest 2. **C,** Abundance of PITG_21410 mRNA based on RNA-seq. The left side of the
814 panel illustrates expression of the gene in hyphae collected from cultures between 3
815 and 6 days after inoculation, and sporangia from a 5-day culture. The right side of the
816 panel shows expression in the 24 RM, TL, PL, and PT sporangia samples.
817
818 **Fig. 4.** Effect of sporangia source on mRNA levels of selected functional groups. **A,** Cell
819 wall degrading enzymes. Sporangia were from harvest 1 tomato leaflets (TL1), potato
820 leaflets (PL1), rye-sucrose media (RM1), and potato tubers (PT1). FPKM levels of
821 genes in each group were added together and normalized to the average of the group.
822 Results from the harvest 2 sporangia were similar. **B,** Metabolic pathways. Samples are

823 the same as those in panel A, and expression levels were normalized to the average of
824 each functional category. **C,** ABC transporters. Indicated are the ratios of mRNA for
825 each gene in sporangia from tomato leaflets (TL1) versus rye media (RM1). **D,** Same as
826 panel C but for RXLRs.
827
828 **Fig. 5.** Sporangia shapes from rye-sucrose media (RM), potato leaflets (PL), and
829 tomato leaflets (TL). Values are based on a minimum of 200 sporangia per sample at
830 the harvest 1 timepoint. Variation was assessed using a paired T-test, and pairs
831 differing at $P<0.01$ are marked by an asterisk.
832
833 **Fig. 6.** Infection assays using zoospores from rye-sucrose media or potato leaflets. **A,**
834 Representative infected leaflets. Each row illustrates the same four leaflets between 5
835 and 8 days after infection. A total of 100 leaflets were used per treatment per
836 experiment. **B,** Percent of leaflets showing infection in three independent experiments.
837 **C,** Average size of lesions in each experiment. **D,** Sporangial density on infected
838 leaflets.
839 **Fig. 7.** Germination rates of sporangia. **A,** Indirect germination of isolates 1306 and
840 88069 measured 2 and 12 hr after sporangia were cold-treated. Sporangia were
841 harvested from 6-day tomato leaflets or rye-sucrose cultures (inoculated by spreading
842 with sporangia). Concentrations adjusted to 10^4/ml prior to the cold-treatment. **B,**
843 Indirect germination of sporangia from isolate 1306 grown on tomato leaflets, potato
844 leaflets, and spread-inoculated rye-sucrose media. Measurements were made 3 and 6-
845 hr after the cold treatment, using sporangia harvested 5, 6, and 8 days after inoculation.

846

847

848 **SUPPLEMENTARY TABLES**

849 Table S1. RNA-seq library statistics.

850 Table S2. Expression levels of *P. infestans* genes (CPM).

851 Table S3. Fold-change, *P*-value, and FDR statistics for genes that showed significant

852 differential expression in comparisons of sporangia from different source tissues or

853 sporangia of different ages.

854 Table S4. Gene Ontology (GO term) over-representation analysis.

855

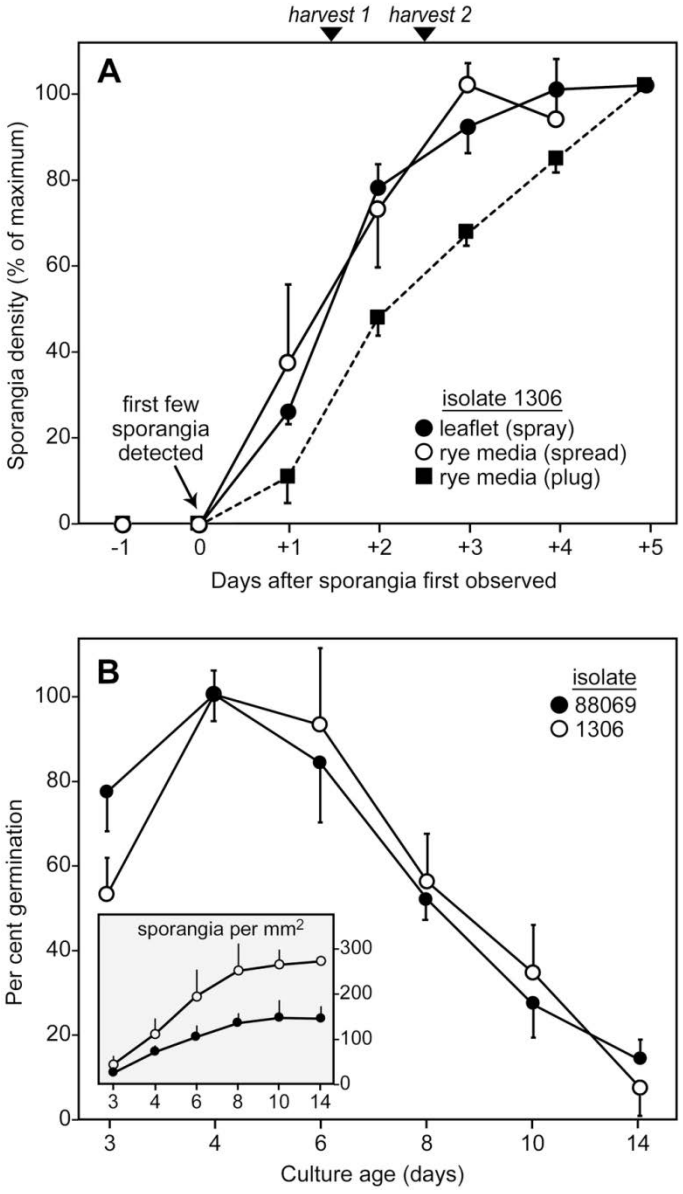

Fig. 1. Influence of age on sporulation and germination in *P. infestans.* A, Time-course of sporulation using isolate 1306. Sporangia were harvested from spray-inoculated tomato leaflets (black circles), rye-sucrose agar inoculated with a lawn of sporangia (open circles), or rye-sucrose agar inoculated 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 percent of maximum, which equaled 710±69, 247±25, and 331±56 sporangia per mm2 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 hr after being placed in Petri's solution at 10 degrees C. The values are expressed relative to the maximum for each isolate, which equaled 82% and 71% germination for isolates 88069 and 1306 on day 4, respectively. 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.

85x150mm (300 x 300 DPI)

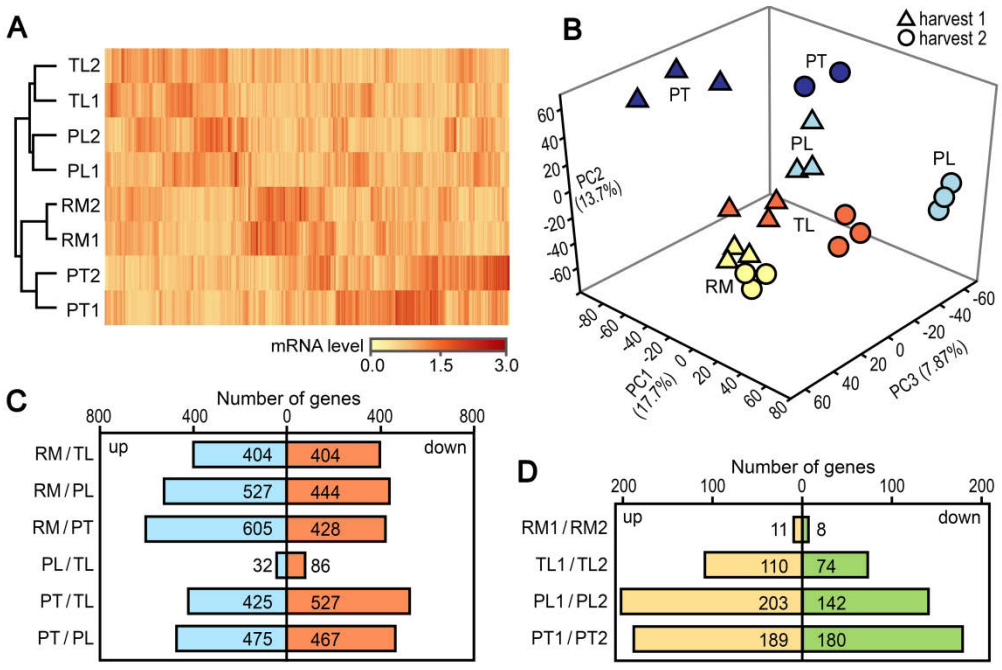

Fig. 2. mRNA levels in sporangia from different culture conditions. A, Heatmap representing mRNA from tomato leaflets (TL), potato leaflets (PL), rye-sucrose media (RM), and potato tubers (PT). Samples were taken at the times labeled "harvest 1" and "harvest 2" in Fig. 1A, representing sporangia on the 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 >2-fold change and FDR<0.05 at the two timepoints. D, Number of genes differentially expressed between sporangia from the harvest 1 and harvest 2 timepoints (i.e. 24 hr apart).

176x116mm (600 x 600 DPI)

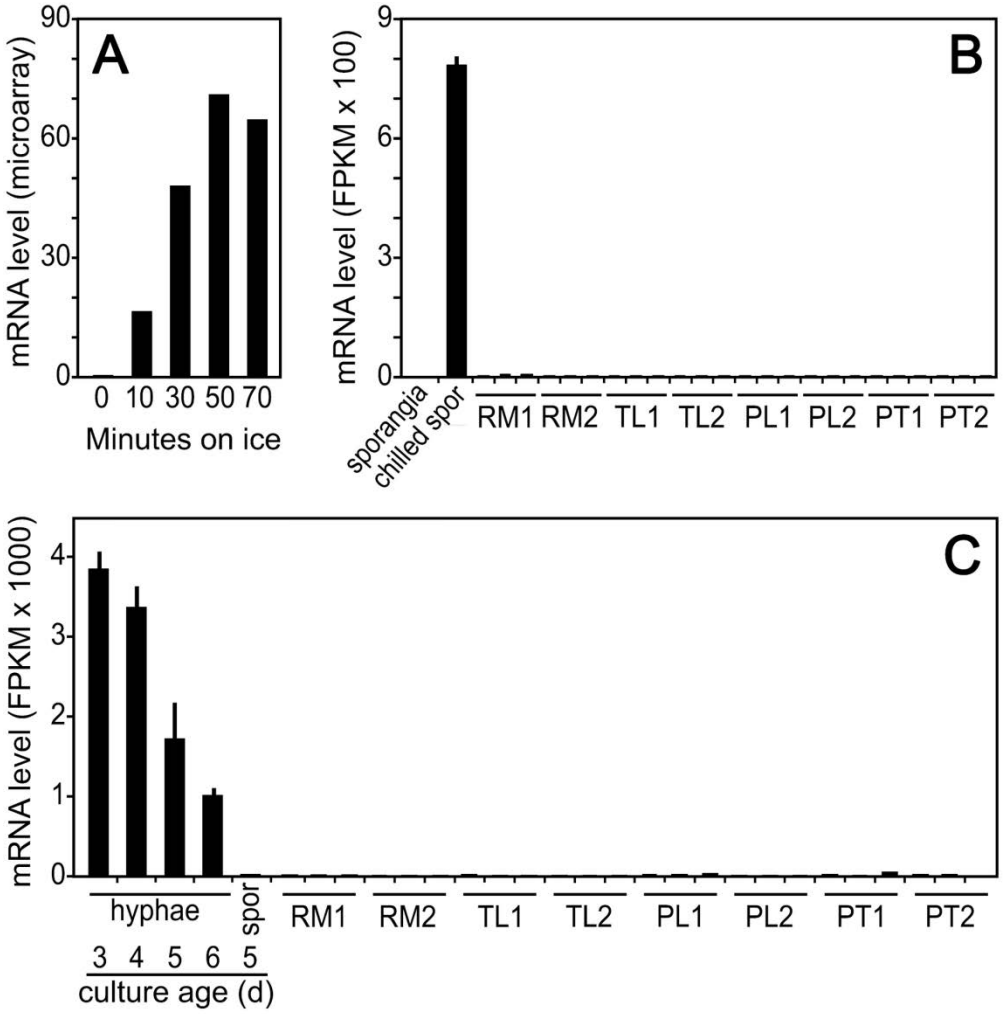

Fig. 3. Quality assessments of the 24 sporangia samples. A, Expression of zoosporogenesis-induced gene PITG_13579 in sporangia immediately after harvesting and after 10, 30, 50, and 70 min of chilling in Petri's solution. Data are based on microarray analysis. B, Abundance of PITG_13579 mRNA based on 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. 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 extracted from a 5-day culture. The right side of the panel shows expression in the 24 RM, TL, PL, and PT sporangia samples.

84x86mm (600 x 600 DPI)

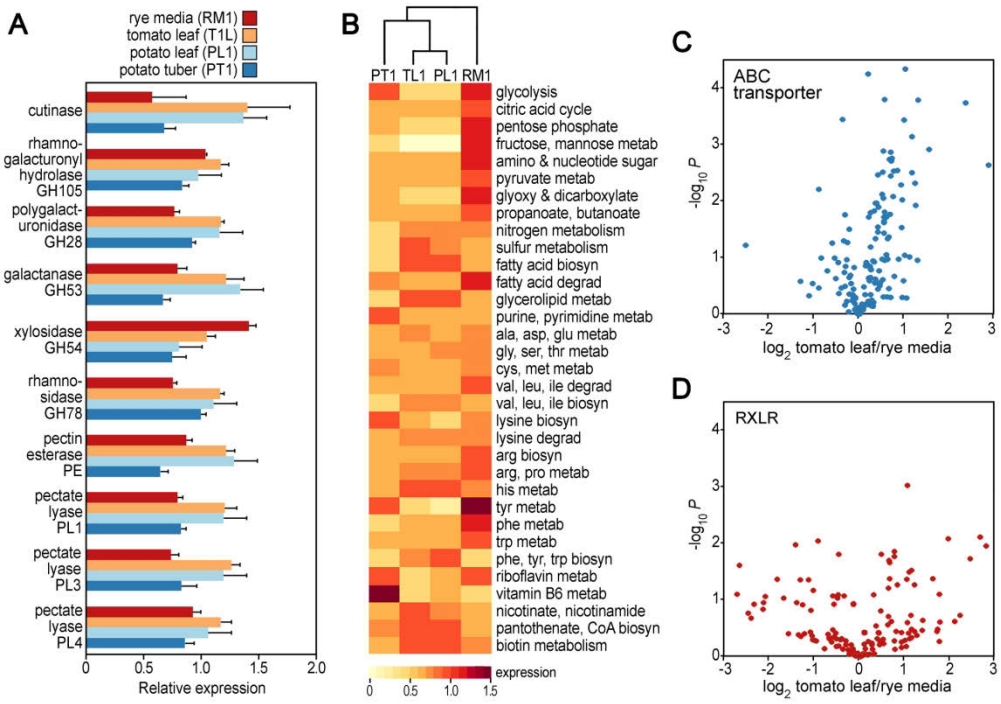

Fig. 4. Effect of sporangia source on mRNA levels of selected functional groups. A, Cell wall degrading enzymes. Sporangia were from harvest 1 tomato leaflets (TL1), potato leaflets (PL1), rye-sucrose media (RM1), and potato tubers (PT1). FPKM levels of genes in each group were added together and normalized to the average of the group. Results from the harvest 2 sporangia were similar. B, Metabolic pathways. Samples are the same as those in panel A, and expression levels are normalized to the average of each functional category. C, ABC transporters. Indicated is the ratio of mRNA for each genes in sporangia from tomato leaflets (TL1) versus rye media (RM1). D, Same as panel C but for RXLRs.

177x125mm (300 x 300 DPI)

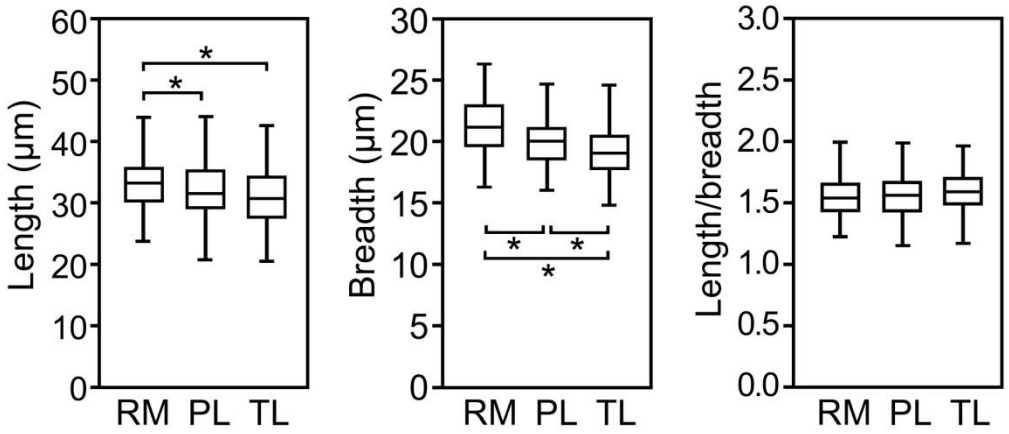

Fig. 5. Sporangia shapes from rye-sucrose media (RM), potato leaflets (PL), and tomato leaflets (TL). Values are based on a minimum of 200 sporangia per sample at the harvest 1 timepoint. Variation was assessed using a paired T-test, and pairs differing at $P<0.01$ are marked by an asterisk.

85x36mm (600 x 600 DPI)

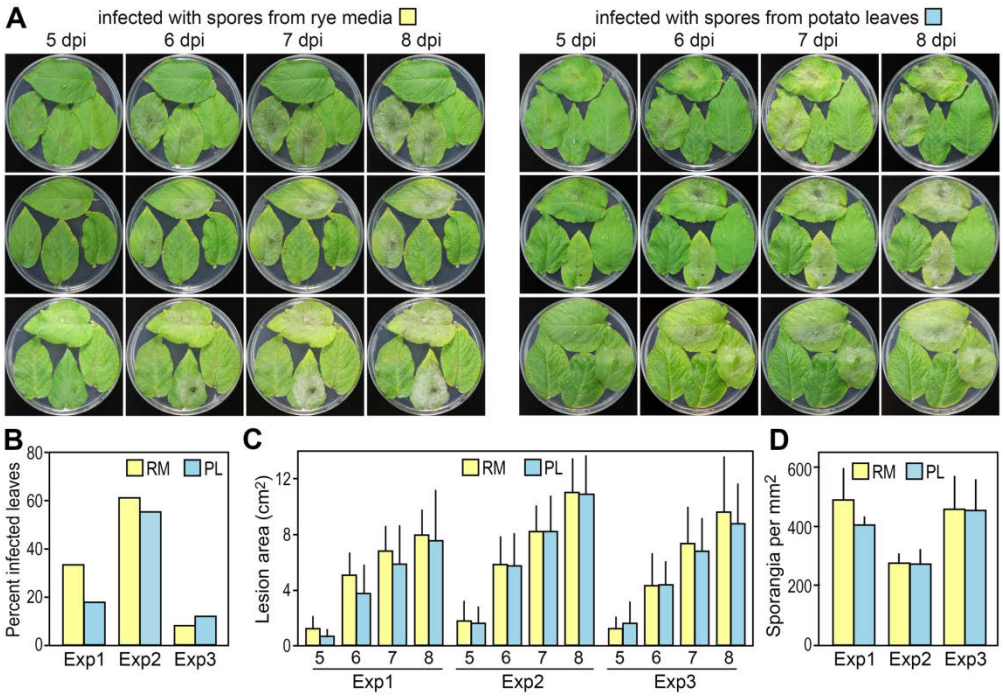

Fig. 6. Infection assays using zoospores from rye-sucrose media or potato leaflets. A, Representative infected leaflets. Each row illustrates the same four leaflets between 5 and 8 days after infection. A total of 100 leaflets were used per treatment per experiment. B, Percent of leaflets showing infection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

177x123mm (300 x 300 DPI)

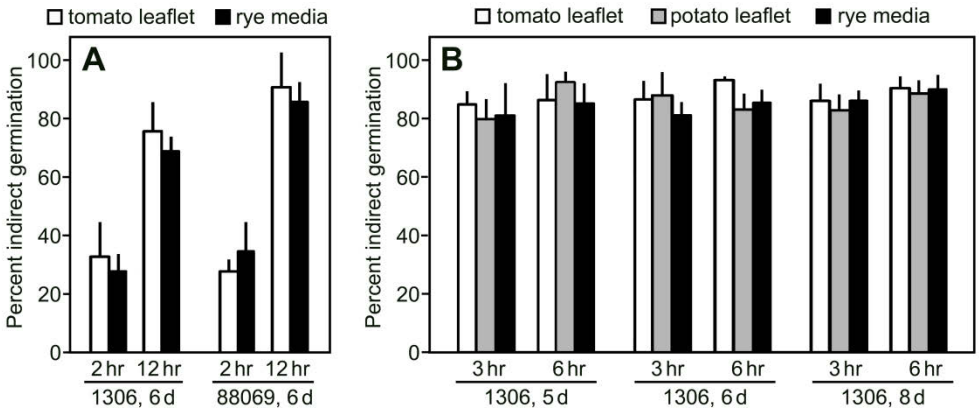

Fig. 7. Germination rates of sporangia. A, Indirect germination of isolates 1306 and 88069 measured 2 and 12 hr after sporangia were cold-treated. Sporangia were harvested from 6-day tomato leaflets or rye-sucrose cultures (inoculated by spreading with sporangia). Concentrations adjusted to 104/ml prior 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-hr after the cold treatment, using sporangia harvested 5, 6, and 8 days after inoculation.

150x62mm (600 x 600 DPI)

Table S1. RNA-seq statistics.

Sample	Replicate number	Numbers of reads	Reads aligned	Percentage aligned
RM1	1	27,786,683	24,964,159	89.84
	2	27,146,322	24,605,862	90.64
	3	24,725,165	22,213,614	89.84
RM2	1	25,276,415	22,753,075	90.02
	2	24,325,007	21,900,093	90.03
	3	23,775,169	21,372,457	89.89
TL1	1	25,782,948	23,315,571	90.43
	2	28,580,358	25,486,716	89.18
	3	26,282,609	23,691,070	90.14
TL2	1	24,050,143	21,882,827	90.99
	2	26,696,667	24,027,537	90.00
	3	30,234,640	26,839,674	88.77
PL1	1	24,423,113	21,240,683	86.97
	2	27,088,531	24,288,373	89.66
	3	33,097,601	30,234,527	91.35
PL2	1	24,704,349	22,168,562	89.74
	2	26,858,060	23,836,667	88.75
	3	25,603,766	23,405,107	91.41
PT1	1	22,888,728	17,742,671	77.52
	2	22,545,273	15,837,798	70.25
	3	22,130,604	12,335,674	55.74
PT2	1	25,510,572	18,569,791	72.79
	2	23,562,556	18,435,517	78.24
	3	25,840,226	20,162,631	78.03