MethodsX 13 (2024) 102980

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/methodsx

Spray inoculation and image analysis-based quantification of powdery mildew disease severity on pea leaves ☆

Poonam Ray, Divya Chandran*

Laboratory of Plant-Microbe Interactions, Regional Centre for Biotechnology, NCR Biotech Science Cluster, Faridabad, 121001, India

ARTICLE INFO

Method name:
Spray inoculation and image analysis-based fungal disease quantification

Keywords:
Erysiphe pisi
Biotrophic fungus
Airbrush
Uniformity index
ImageJ
Semi-automatic
Fungal area
Tubulin

ABSTRACT

Pea (*Pisum sativum*) is an important agricultural legume crop, but powdery mildew disease caused by the biotrophic fungus *Erysiphe pisi* regularly limits its annual yield. Assays to evaluate the efficacy of potential antifungal compounds or resistance genes for disease control require a simple fungal inoculation method that provides control over the initial inoculum concentration and enables uniform inoculum distribution within a leaf and across replicates as well as a method for the quantitative assessment of disease severity. Here, we present an easy spray inoculation method for the uniform distribution of a defined concentration of *E. pisi* conidia on the leaves of pea plants and a semi-automated image analysis-based quantification of disease symptoms. The uniformity in conidial distribution was validated using a novel grading system termed the uniformity index. In addition, RT-qPCR was used to validate the reproducibility of the spray inoculation method and image analysis-based disease quantification. These procedures permit the accurate quantification of powdery mildew disease severity at macroscopic and molecular levels.

- Uniform and reproducible inoculum distribution on leaves using a simple and inexpensive spray device
- Rapid and reproducible quantification of powdery mildew disease symptoms using open-source software without the requirement of computational expertise

Specifications table

Subject area:	Agricultural and Biological Sciences
More specific subject area:	*Plant-pathogen interactions*
Name of your method:	Spray inoculation and image analysis-based fungal disease quantification
Name and reference of original method:	N/A
Resource availability:	ImageJ software (https://imagej.net/ij/; v 1.54f)

Background

Powdery mildew caused by the obligate biotrophic fungal pathogen *Erysiphe pisi* is a devastating disease that limits the productivity of leguminous crops, including pea, lentils, and Medicago species [1]. The fungus infects only the aerial parts of the plant, particularly the leaves, and produces white powder-like disease symptoms, representing the epiphytic mycelia and asexual reproductive structures.

☆ **Related research article:** None

* Corresponding author.
E-mail address: divya.chandran@rcb.res.in (D. Chandran).
Social media: (D. Chandran)

https://doi.org/10.1016/j.mex.2024.102980
Received 28 June 2024; Accepted 24 September 2024
Available online 25 September 2024
2215-0161/© 2024 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/)

A fundamental requirement of powdery mildew studies is a pathogen inoculation method that is simple and reproducible, provides control over the initial inoculum concentration, and ensures uniform conidia distribution across the plant surface so that fungal growth stages and visual disease symptoms can be precisely quantified at microscopic and macroscopic levels, respectively.

The conventional methods for powdery mildew inoculation include tapping or brushing conidia from a heavily infected leaf directly on a plant (e.g., [2]) and dusting conidia over a nylon mesh fixed on a settling tower and allowing the conidia to slowly settle on the plant by gravity (e.g., [3-6]). While the tapping and brushing methods are easy to perform and do not require special equipment, they do not offer any control over the initial inoculum concentration and result in uneven distribution and clumping of conidia, reducing the number of isolated colonies for accurate quantification of fungal growth stages. The settling tower method mimics the natural infection process and produces isolated colonies but extends minimal control over the initial inoculum concentration and requires large amounts of inoculum for infection (typically one to two fully infected leaves per tower) [5].

The liquid-based spray method, in which a conidial suspension is applied over the plant using a fine-spray apparatus, is an alternate but less commonly used powdery mildew inoculation method that offers several advantages over the conventional methods. First, the inoculum concentration can be defined by the number of conidia/ml, enabling reproducibility across replicates. Second, the spray droplet size can be controlled using a fine spray nozzle and surfactants like Tween 20 [7], facilitating uniform distribution of the inoculum across the leaf surface. Third, the spray method requires far less inoculum than the other inoculation methods, with conidia from a single leaf sufficient to inoculate multiple plants. Previously developed spray inoculation methods either use specialized devices that are not easy to replicate and/or are primarily suitable for the inoculation of excised leaf segments, not intact plants [8-11]. Further, the Tween 20 concentration has to be carefully selected as it can adversely impact fungal germination [12].

In the present study, we aimed to develop a liquid-based spray inoculation method that uses a simple and inexpensive air spray device to provide uniform and reproducible distribution of *E. pisi* conidia on the leaves of intact pea plants. We also aimed to develop an image analysis-based quantification method to assess powdery mildew disease severity on pea leaves. Unlike earlier image-based disease quantification methods that use complicated algorithms (e.g., [13-16], our goal was to develop a simple method that uses open-source software and does not require any special computational expertise.

Method details

Plant and fungal material

Pea (*Pisum sativum*) cv. AP3 plants were grown at 22 ˚C in a controlled environment growth chamber (Conviron, Canada) with a 16-h light/8-h dark cycle, 70% humidity, and photosynthetically active radiation of 170 μmol m^{-2} s^{-1}. Pure cultures of *Erysiphe pisi* (Palampur-1 isolate; https://mycocosm.jgi.doe.gov/Erypi2/Erypi2.home.html) were maintained on AP3 pea plants grown in a separate Conviron growth chamber under the above-mentioned environmental conditions.

Preparation of E. pisi *conidial suspensions for spray inoculation*

E. pisi conidia from an infected pea leaf showing white powder-like symptoms [12–14 days post inoculation (dpi)] was brushed into 20 ml of 0.02 %, 0.03 %, 0.04 %, or 0.1 % Tween 20 solution. Each suspension was briefly vortexed to disperse the conidia in the solution, centrifuged for 5 min at 4000 rpm to dislodge any conidia stuck to the tube walls, and then remixed by gentle pipetting to uniformly disperse the conidia in the Tween 20 solution. The number of conidia in each suspension was counted using a haemocytometer (Bürker-Türk, Ref-719505, Germany). A typical haemocytometer has two counting chambers, each with 9 square grids (Fig. 1). A coverslip was placed on the counting chambers, and 10 μl of the conidial suspension was slowly dispensed into each chamber through the gap between the coverslip and the slide using a micropipette. The haemocytometer was then observed under the 10X objective of a light microscope (Olympus CX-23, Japan). The four corner grids with the larger squares were used for conidia counting. Conidia counts from at least three technical replicates were averaged and used to estimate the final conidia concentration using the following equation: Number of conidia/ml = Average number of conidia per grid/volume of 1 grid (ml); where the volume of 1 grid = 0.1 cm × 0.1 cm × 0.01 cm = 0.0001 cm^3 = 0.0001 ml. The suspensions were diluted to 10,000 conidia/ml using the Tween 20 solution before inoculation. Higher conidia/ml concentrations can be used for inoculation but may not be optimal for the quantification of fungal growth stages, which require isolated colonies. Conidial suspensions may be stored at room temperature for up to 10 h without losing viability.

Spray inoculation of E. pisi *conidia*

Pea plants with 3–4 pairs of fully expanded mature leaves (~20 days old) were spray inoculated with the *E. pisi* conidial suspension using an airbrush [Zorbes Spirit Air 0.4 mm Multi-Purpose Airbrush Compressor Spray) (Fig. 2)]. The leaves were sprayed from a 7–8 cm distance using the lowest airflow speed setting (Fig. 2b). After spraying, the plants were allowed to dry for an hour, covered with a dome to maintain humidity (Fig. 2c), and placed in the dark for 24 h to facilitate conidia germination (Fig. 2d). The sprayed leaves were harvested at different time points after inoculation to calculate the conidia uniformity index, and % conidia germination, and to quantify the fungal load via image and gene expression-based analyses.

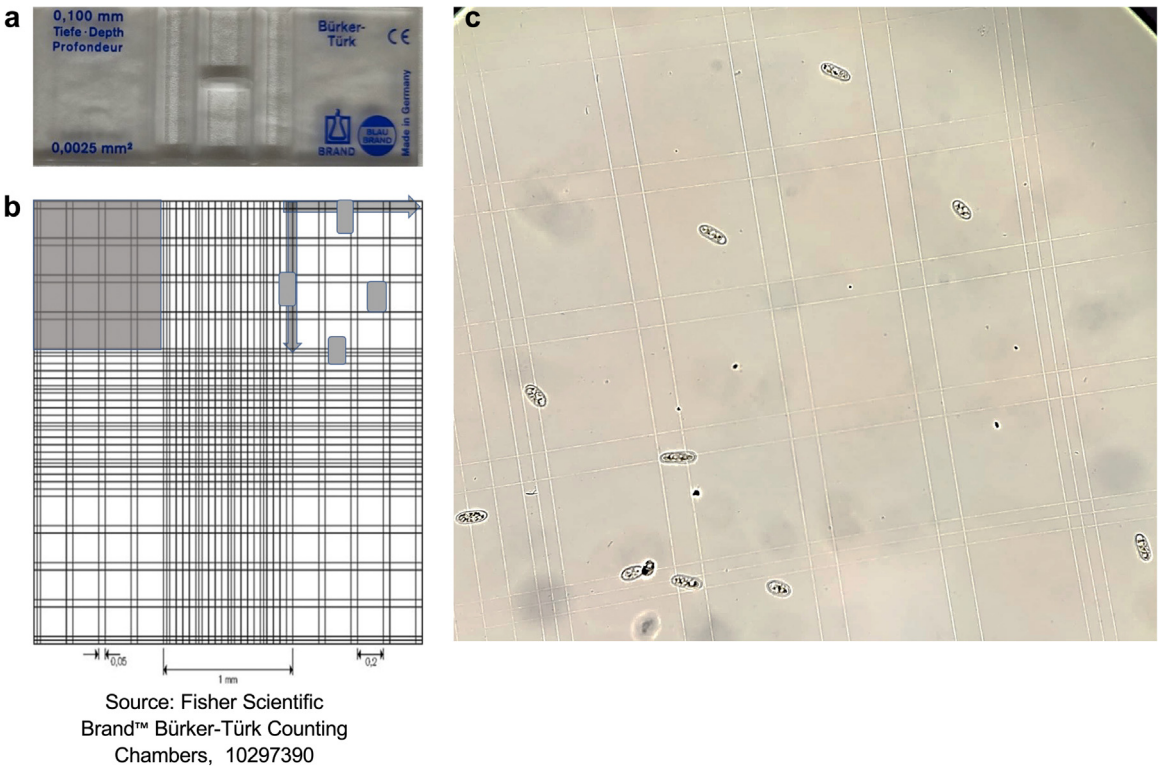

Fig. 1. Conidia counting chamber used to estimate initial inoculum concentration a) A typical counting chamber or haemocytometer with two grid chambers separated by a horizontal line b) A zoomed image of the haemocytometer grid with 9 large squares (one highlighted in grey) and each large square containing 16 small squares c) Conidia present within the large square separated by triple line should be counted.

Trypan blue staining and microscopy

One-half of each infected leaf was cleared in 95 % ethanol and stained with 250 μg/ml trypan blue in a solution of lactic acid, glycerol, and water (1:1:1) for 15 min. The samples were rinsed with the same solution and mounted with 50 % glycerol on glass slides [17]. The stained leaf sections were observed under 50X magnification in a brightfield Zeiss Observer.Z1-AXIO microscope (Carl Zeiss, Germany).

Calculation of conidia uniformity index (UI) and percent germination

We developed a grading system to evaluate the uniformity of conidia distribution on the leaf surface after spray inoculation. A field of view (FOV) under a 50X magnification of the Zeiss Observer1.Z1 is a rectangular-shaped area of 0.06 cm^2. We estimated that a 3–4 cm^2 area half-leaf section would contain ~50–70 FOV. Based on the initial inoculum concentration of 10,000 conidia/ml and ~70–100 μl of spray volume per leaf, we estimated that, under optimal conditions, an entire leaf would receive ~700–1000 conidia and a half-leaf would receive ~350–500 conidia. If the inoculum is distributed uniformly on the leaf surface, we expect to obtain 5–20 conidia/FOV. In addition to the number of conidia, the distance between conidia within a FOV was also considered in the grading system. At least 30 FOV that contained conidia were captured per leaf section at 1 dpi. In the leaf samples sprayed with 0.002 % and 0.003 % Tween 20 suspensions, all FOV that contained conidia were captured as there were fewer than 30. From these FOV, five images were arbitrarily selected for each Tween 20 concentration, and the number of conidia and distance between conidia were measured. Based on this information, a grade between 1 and 10 was assigned to each FOV, as detailed in Table 1. These grades were used to calculate a Uniformity Index (UI) with the formula:

[Total number of FOV with conidia/Total number of FOV in half-leaf] x FOV grade average/leaf section UI ≥ 7 was considered a highly uniform distribution of inoculum; UI between 4 and 7 was uniform; 1 to 4 was less uniform; and 0 to 1 was non-uniform. The number of germinated conidia at the different Tween 20 concentrations was determined at 2 dpi from three sets of independent experiments.

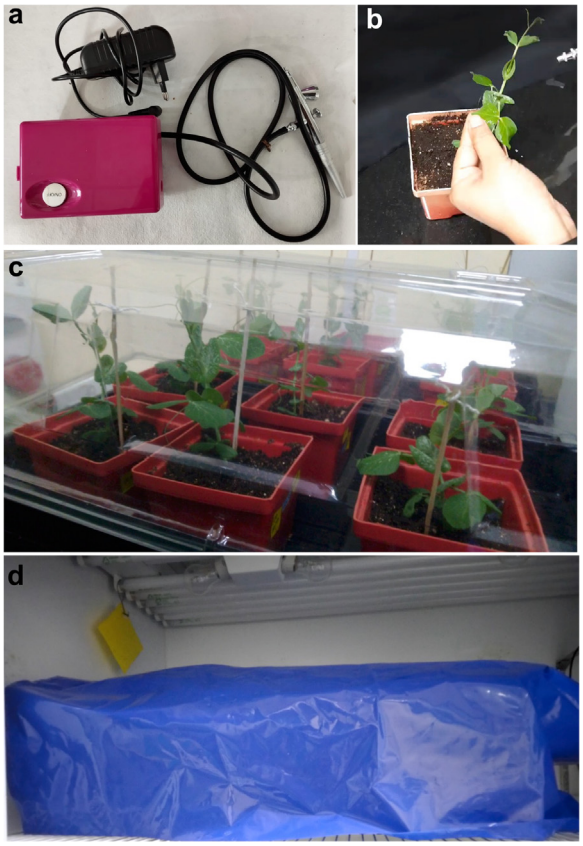

Fig. 2. Spray inoculation using airbrush and plant setup a) Airbrush used for spray inoculation with optional settings for three air flow rates b) Pea leaves sprayed with *E. pisi* conidia using the lowest air flow rate setting c) Plants covered with a plastic dome after spray inoculation, and d) placed in the dark for 24 h.

Table 1
Grading system used for calculating the conidia uniformity index.

No. of conidia	Distribution condition in a focal area	Negative marking of 0.1 for each pair of conidia < 0.2 mm apart	Grade
5 to 20	All separate (>=0.2 mm apart)	0	10
	10 pairs	10+10 x (−0.1)	9
	5 pairs	10+5 x (−0.1)	9.5
	3 pairs	10+3 x (−0.1)	9.7
	All clumped	1/10th of 'all separate' grade	1
1 to 4	All separate	0	5
	2 pairs	5 + 2 x (−0.1)	4.8
	All clumped	1/10th of 'all separate' grade	0.5
21 to 40	All separate	0	5
	4 pairs	5 + 4 x (−0.1)	3
	All clumped	1/10th of 'all separate' grade	0.5
>40	All separate	0	2.5
	20 pairs	2.5 + 20 x (−0.1)	0.5
	All clumped	1/10th of 'all separate' grade	0.25

ImageJ-based quantification of powdery mildew disease symptoms and spray droplet size

The open-source software, ImageJ (https://imagej.net/ij/; version 1.54f) was used for the quantification of powdery mildew disease symptoms. A previously established ImageJ protocol, designed to quantify bacterial colony size on media plates [18-19], was modified to quantify the area covered by powdery mildew colonies on pea leaves (Fig. 3). The infected pea leaf and a physical ruler placed in the same frame were photographed using a digital camera (NIKON D3500 DSLR). The scale of the image was set by opening the captured image in ImageJ and converting it into the 8-bit format. The "Straight" line tool was selected from the toolbox, and a line was drawn on the ruler covering a distance of 1 cm. The scale was set in the "Analyze" tab by changing the unit to cm and

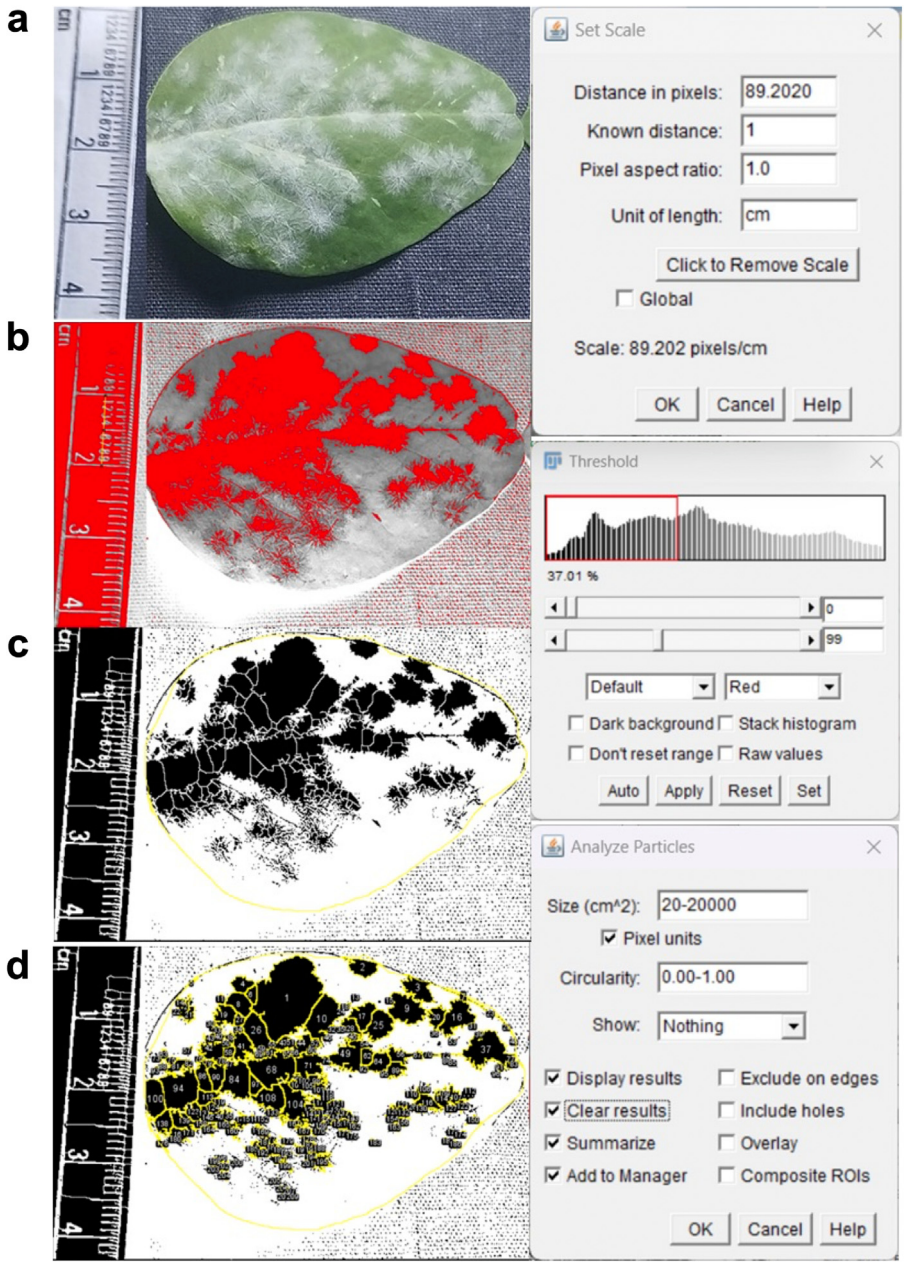

Fig. 3. Fungal area quantification using ImageJ a) Leaf image selected for fungal area measurement showing scale set to 1 cm based on the white ruler photographed in the same frame b) Threshold auto-set between 0 and 255 as per the image pixel quality c) The Watershed feature delineates the fungal area from the leaf background (outlined in yellow). d) Individual areas (black with yellow outline) are indexed and measured.

entering the length (1 cm) selected on the physical ruler. In the “Edit” tab, the image was inverted, and in the “Process” tab, the “enhance contrast” option was adjusted to 75 %. The threshold of the image was adjusted between 0 and 255 in the “Image>Adjust” tab so that only the fungal area was selected. The “watershed” function under the “Process>Binary” was selected to delineate the region of interest (entire leaf) using the “freehand selection” tool. The “Size” tab under the “Analyze Particles” tool was set to 20 to 20,000 pixels to capture the maximum fungal area. We recommend optimizing the pixel range for each application as it depends on the camera quality and the area of the region of interest being quantified. In the ‘Analyze Particles’ tab, the “Pixel units” and “Summarize” options were selected to convert the total number of pixels covering the fungal area into cm^2, and the percent leaf area covered by the fungus was obtained. A macro was created for this analysis pipeline to run as a semi-automated plugin function in ImageJ (Supplementary File S1). The spray droplet size and number were also measured using ImageJ, as described above.

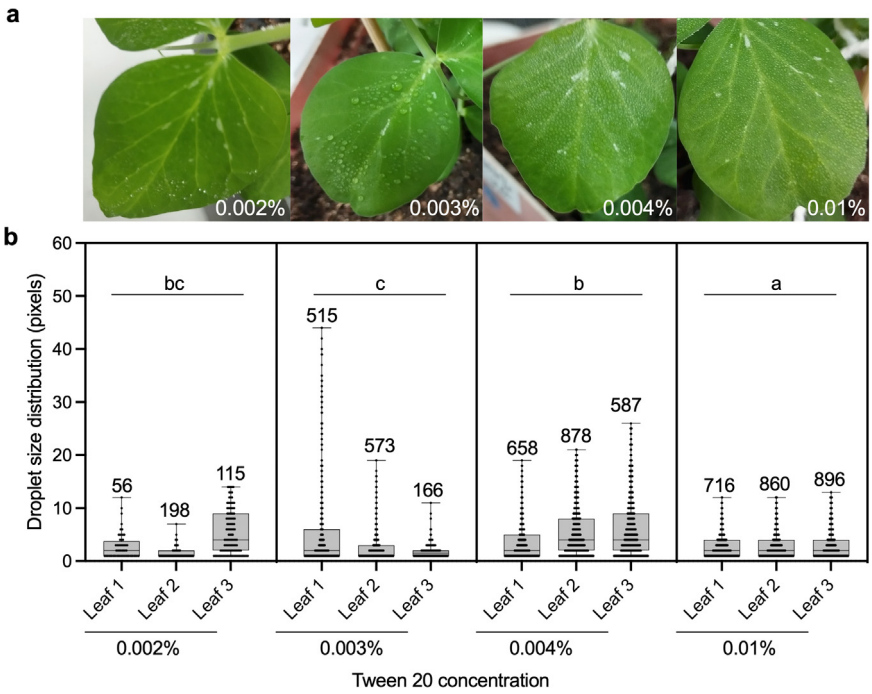

Fig. 4. Effect of Tween 20 concentration on inoculum droplet size a) Appearance of droplets on pea leaf sprayed with different Tween 20 solutions b) Box and whiskers plot showing the distribution of spray droplet size and total number of droplets (provided above each box) per leaf for three leaves per treatment. Statistical significance between treatments was computed using One-way ANOVA ($p < 0.0001$) along with Tukey's multiple comparisons tests ($\alpha < 0.05$) and is shown by different lower-case letters.

RNA extraction, cDNA synthesis, and RT-qPCR for fungal quantification

Infected pea leaves (100 mg) were ground in liquid nitrogen, and total RNA was extracted using the Nucleospin RNA Plant kit (Macherey-Nagel, Germany) with on-column DNase treatment per the manufacturer's protocol. 1.5 µg of total RNA was used to synthesize first-strand cDNA using the PrimeScript cDNA synthesis kit (Takara, Japan) per the manufacturer's protocol. Two-fold diluted cDNA was used for RT-qPCR using the 5X TB Green Premix qPCR mix (Takara-Bio Inc.) in a QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems, USA). Three biological replicates from three independent experiments were processed. Pea *β-tubulin 2* (NCBI accession ID: X54845.1) was used as the endogenous control. Relative expression of *E. pisi β-tubulin 2* (NCBI accession ID: X81961.1) was calculated using LinRegPCR v. 2021.2 [20]. The primer list is provided in Table S1.

Method validation

One of the major factors affecting spray droplet size and number, as well as the uniformity of conidia distribution on the leaf surface, is the concentration of the surfactant used in the spray suspension. To determine the effect of Tween 20 concentration on the uniformity and reproducibility of the spray across leaf samples, we measured the number and size of the spray droplets on the surface of pea leaves (Fig. 4a). The droplet number was higher and relatively more uniform across leaf samples at the higher Tween 20 concentrations (0.01 % and 0.004 %) than in the lower concentrations (0.002 % and 0.003 %) (Fig. 4b). Further, although, smaller droplets were observed at the lowest (0.002 %) and highest (0.01 %) Tween 20 concentrations than at the other concentrations (Fig.4b), the uniformity in droplet size across replicates was only observed in the 0.01 % Tween 20-sprayed leaves (Fig. 4b). This is likely because the larger droplets in the 0.002 % Tween 20-sprayed leaves do not adhere to the leaf surface and roll off, leaving behind the smaller droplets (Fig. 4a). Additionally, even though the 0.004 % Tween 20-sprayed leaves were effective in holding a large number of droplets, the droplet size distribution was not as uniform across replicates as on the 0.01 % Tween 20-sprayed leaves. Therefore, we observed the highest uniformity in droplet size and number in the leaves sprayed with the 0.01 % Tween 20 solution.

To determine the effect of Tween 20 on the uniformity of inoculum distribution within and across leaf samples, we sprayed pea leaves with *E. pisi* conidial suspensions containing 10,000 conidia/ml in different Tween 20 concentrations. Our preliminary experiments with a range of conidia concentrations (10,000 to 100,000/ml) showed that 10,000 conidia/ml provided the optimal number to assess the uniformity of inoculum distribution and produce isolated colonies for disease symptom quantification (data not shown). At 1 dpi, the sprayed pea leaves were harvested, and one-half was stained with trypan blue to visualize the fungal structures under a microscope. We observed that with increasing Tween 20 concentration, the *E. pisi* conidia appeared more dispersed and less clumped on the leaf surface (Fig. 5a). We used the Uniformity Index (UI) grading system developed in this study to quantify this

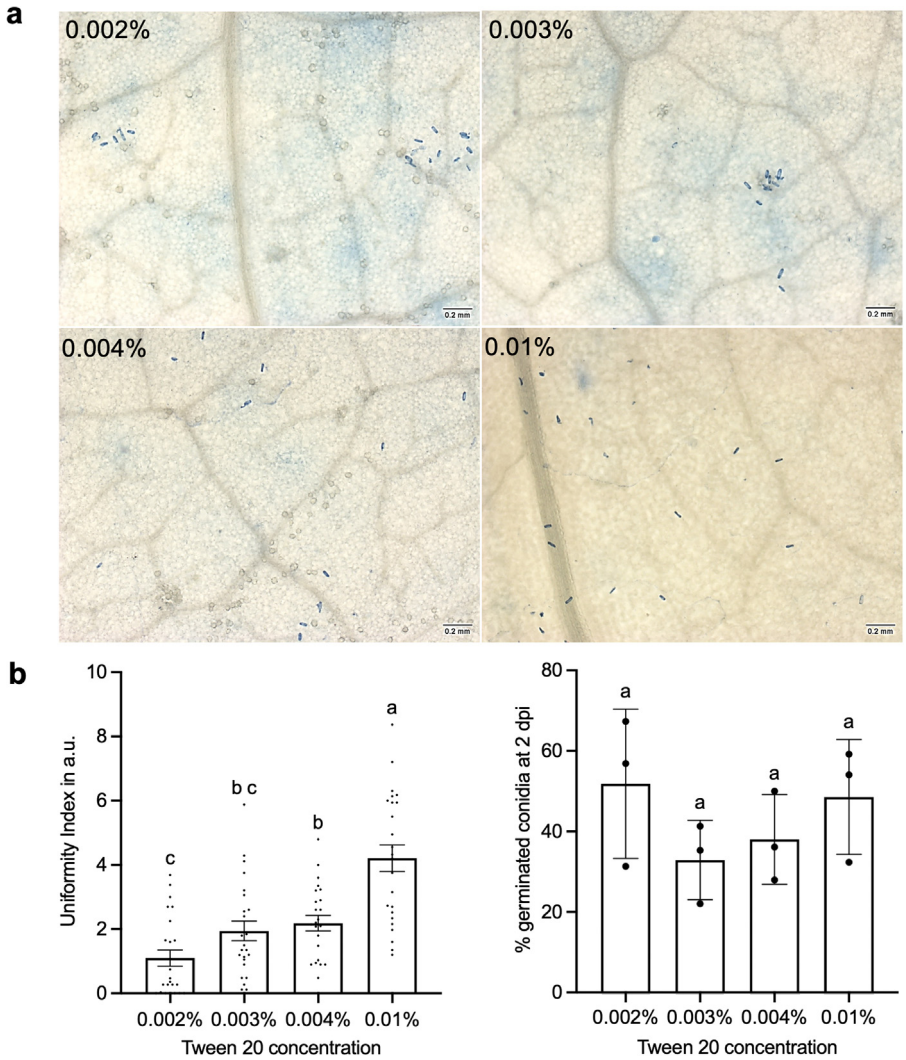

Fig. 5. Effect of Tween 20 concentration on conidia distribution and germination a) Distribution of *E. pisi* conidia on pea leaf surface visualized by trypan blue staining after spray inoculation with conidial suspension containing 0.002 %, 0.003 %, 0.004 %, or 0.01 % Tween 20 b) Bar graph showing the mean uniformity index (±SEM) calculated based on the distribution of conidia in five FOV from eight leaves per Tween 20 treatment at 1 dpi. Statistical significance between treatments was computed using Brown-Forsythe and Welch-ANOVA tests ($p < 0.0001$) along with Dunnett's T3 multiple comparison test ($\alpha < 0.05$) and is shown by different lower-case letters. c) Bar graph showing% germinated conidia at 2 dpi per leaf for three leaves per Tween 20 treatment. Statistical significance between treatments was computed using One-way ANOVA and Tukey's multiple comparisons tests. Data was not significant.

observation. Based on our grading system, a UI value between 4 and 7 indicates a uniform distribution of conidia. The average UI for the 0.01 % Tween 20-sprayed leaves was 4.2, significantly higher than the UI values (1.1–2.2) obtained for the other Tween 20 concentrations (Fig. 5b). To test whether Tween 20 affects the viability of *E. pisi* conidia, we calculated the percent of germinated conidia at 2 dpi after spray inoculation. We did not observe a significant difference in conidia germination on leaves sprayed with suspensions prepared in different Tween 20 concentrations (Fig. 5c). The UI and conidia germination results confirm that conidial suspensions made with 0.01 % Tween 20 provide an even distribution of inoculum across the leaf surface.

Reproducibility of the spray inoculation method, in terms of the initial amount of inoculum deposited on the leaf, is critical, mainly when the goal is to compare the degree of infection between plant genotypes or treatments. To validate the reproducibility of our method, we photographed pea leaves spray-inoculated with 10,000 conidia/ml suspensions (0.01 % Tween 20) at 7 dpi, when white powder-like powdery mildew symptoms were visible on the pea leaf surface. We used the ImageJ software and the semi-automated image analysis-based quantification plug-in developed in this study to quantify the percent of leaf area covered by the disease (Fig. 6a). The image-based quantification revealed that ~40–50 % of the leaf area was covered with white powder-like symptoms in leaf samples from two independent experiments (Fig. 6b). While experiment 2 replicate values were highly similar, two of the four replicates in experiment 1 showed a slight deviation from the mean. This discrepancy may be attributed to the inherent

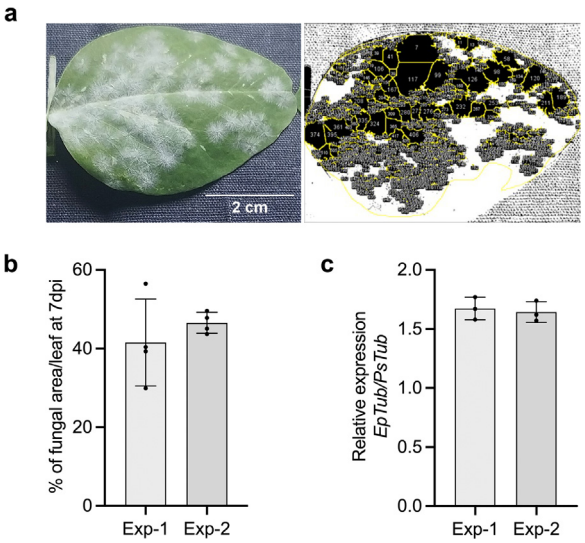

Fig. 6. Quantification of powdery mildew disease symptoms via image-based analysis and RT-qPCR of fungal gene a) Left panel: Infected pea leaf showing white colonies of powdery mildew at 7 dpi with 10,000 conidia/ml in 0.01 % Tween 20; Right panel: Region of Interest (ROI) selected by the ImageJ plug-in for quantification of fungal area b) Bar graph showing mean (±SD)% pea leaf area covered by powdery mildew calculated using ImageJ from 4 leaves each from two independent experiments c) Bar graph showing mean relative expression (±SD) of *EpTub* (*E. pisi tubulin)* normalized to *PsTub* (*P. sativum tubulin*) at 7 dpi after spray inoculation from 3 biological replicates and two independent experiments.

biological variation in powdery mildew growth across replicate leaf samples or to the inability of the ImageJ software to capture all infected areas accurately. To further assess the reproducibility of the spray inoculation method, we determined the fungal load on the leaf at 7 dpi through RT-qPCR expression analysis of *E. pisi tubulin*. We found similar levels of *Eptubulin* expression across biological replicates within an experiment and across experiments (Fig. 6c), demonstrating the reproducibility of the spray inoculation method.

Our spray inoculation method represents a significant improvement over earlier methods [4,16] in terms of simplicity, cost, speed, and application, as it does not require any specialized device, and is suitable for the inoculation of excised leaf segments as well as intact plants. In terms of cost, we estimate a one-time investment of ~$100 for the simple airbrush (~$60) and haemocytometer ($30-$50) used in the spray inoculation method reported here. The images for the ImageJ-based powdery mildew disease quantification can be captured on a regular phone camera. In terms of time, the entire process, from conidia counting to spray inoculation, can be achieved in <1 hour, and by following the simple on-screen instructions, a relatively inexperienced ImageJ user will be able to perform the semi-automated disease symptom quantification method at the rate of ~ 1–2 min per leaf. Furthermore, in terms of application, this method is especially suitable for studies comparing disease severity on wild-type and mutant plants or after treatment with different antifungal compounds where tight control over the initial inoculum density is essential. In addition, an even distribution of conidia over the leaf surface without clumping facilitates the development of isolated colonies, permitting accurate quantification of fungal growth stages. Our quick and reproducible inoculation and quantification method can be easily extended to powdery mildews infecting other plant species.

Limitations

Using our simple spray inoculation method, we were able to achieve moderate uniformity in inoculum distribution (average UI>4) across the pea leaf with *E. pisi* conidial suspensions prepared in 0.01 % Tween 20. However, if cost is not an issue, it may be possible to achieve higher uniformity by using more sophisticated airbrush devices that can reproducibly dispense a desired volume of liquid per spray at a particular pressure [21]. In addition, while Tween 20 concentrations up to 0.01 % did not significantly impact conidia germination, the germination rate of *E. pisi* conidia is slower after spray inoculation compared to the settling tower and brushing methods. Therefore, the infection time points used to assess disease symptoms will have to be adjusted accordingly.

Ethics statements

N/A

CRediT author statement

Poonam Ray: Conceptualization, Methodology, Validity tests, Data Analysis, Writing – Original draft preparation. **Divya Chandran:** Conceptualization, Supervision, Writing- Reviewing and Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We thank Akriti Sharma, Kesiraju Karthik, Chetan Chauhan, Ankita Alexandar, and Debashish Sahu for critical feedback on the grading system used to calculate the Uniformity Index. We also thank Gulshan Thakur for assistance with conidia counting and Rabishankar Ohja, Aryan, Sumit Sagar, and Smritilekha Mukherjee for help with photography.

This work was supported by a DBT grant (BT/PR36172/NNT/28/1811/2021) to DC and UGC Fellowship to PR.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2024.102980.

References

[1] S. Fondevilla, D. Rubiales, Powdery mildew control in pea. A review, Agron. Sustain. Dev. 32 (2011) 401–409.
[2] A. Sharma, D. Chandran, Host nuclear repositioning and actin polarization towards the site of penetration precedes fungal ingress during compatible pea-powdery mildew interactions, Planta 256 (2022) 45.
[3] R. Wessling, R. Panstruga, Rapid quantification of plant-powdery mildew interactions by qPCR and conidiospore counts, Plant Methods 8 (2012) 35.
[4] R. Delventhal, M. Loehrer, D. Weidenbach, U. Schaffrath, Inoculation of rice with different pathogens: sheath blight (*Rhizoctonia solani*), damping off disease (*Pythium graminicola*) and barley powdery mildew (*Blumeria graminis* f. sp. *hordei*), Bio-Protoc. 6 (2016).
[5] M. Gupta, G. Sharma, D. Saxena, R. Budhwar, M. Vasudevan, V. Gupta, et al., Dual RNA-Seq analysis of *Medicago truncatula* and the pea powdery mildew *Erysiphe pisi* uncovers distinct host transcriptional signatures during incompatible and compatible interactions and pathogen effector candidates, Genomics 112 (2020) 2130–2145.
[6] Y. Wu, D. Diaz, J. Yin, D. Bloodgood, W. Sexton, C.I. Wei, S. Xiao, An easy and flexible inoculation method for accurately assessing powdery mildew-infection phenotypes of Arabidopsis and other plants, J. Vis. Exp. (2021).
[7] K. Arand, E. Asmus, C. Popp, D. Schneider, M. Riederer, The mode of action of adjuvants-relevance of physicochemical properties for effects on the foliar application, cuticular permeability, and greenhouse performance of pinoxaden, J. Agric. Food Chem. 66 (2018) 5770–5777.
[8] P.H.D Reeser, D.I Rouse, Quantitative inoculations with *Erysiphe pisi* to asseess variation of infection efficiency on peas, Phytopathol 73 (1983) 1238.
[9] A.B.M.N.U. Chowdhury, G.B. Bremer, D.W. Salt, P. Miller, M.G. Ford, A novel method of delivering *Blumeria graminis* f. sp. *hordei* spores for laboratory experiments, Crop. Prot. 22 (2003) 917–922.
[10] A.-S. Miclot, S. Wiedemann-Merdinoglu, E. Duchêne, D. Merdinoglu, P Mestre, A standardised method for the quantitative analysis of resistance to grapevine powdery mildew, Eur. J. Plant Pathol. 133 (2012) 483–495.
[11] M.A. Azmat, A.A. Khan, A. Saeed, M. Ashraf, S. Niaz, Screening pea germplasm against *Erysiphe polygoni* for disease severity and latent period, Int. J. Veg. Sci. 18 (2012) 153–160.
[12] L.A. Mwamburi, M.D. Laing, R.M. Miller, Effect of surfactants and temperature on germination and vegetative growth of *Beauveria bassiana*, Braz. J. Microbiol. 46 (2015) 67–74.
[13] N. Sengar, M.K. Dutta, C.M. Travieso, Computer vision-based technique for identification and quantification of powdery mildew disease in cherry leaves, Computing 100 (2018) 1189–1201.
[14] K. Lin, L. Gong, Y. Huang, C. Liu, J. Pan, Deep learning-based segmentation and quantification of cucumber powdery mildew using convolutional neural network, Front. Plant Sci. 10 (2019) 155.
[15] T. Qiu, A. Underhill, S. Sapkota, L. Cadle-Davidson, Y. Jiang, High throughput saliency-based quantification of grape powdery mildew at the microscopic level for disease resistance breeding, Hortic. Res. 9 (2022) uhac187.
[16] A.A.I. Eldesoky, H.M. Abd El-Nabi, E.-S.E Omran, Detecting mildew diseases in cucumber using image processing technique, Misr J. Agric. Eng. 40 (2023) 243.
[17] L. Adam, S.C. Somerville, Genetic characterization of five powdery mildew disease resistance loci in *Arabidopsis thaliana*, Plant J 9 (1996) 341–356.
[18] C. Guzman, M. Bagga, A. Kaur, J. Westermarck, D. Abankwa, ColonyArea: an ImageJ plugin to automatically quantify colony formation in clonogenic assays, PLoS ONE 9 (2014) e92444.
[19] Harper J. ImageJ plate count protocol. protocols.io. 2016.
[20] J.M. Ruijter, C. Ramakers, W.M. Hoogaars, Y. Karlen, O. Bakker, M.J. van den Hoff, A.F. Moorman, Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data, Nucleic Acids Res. 37 (2009) e45.
[21] X. Jin, Y. Huang, S.J. Thomson, R.B. Elliott, Effects of conidial densities and spray volume of *Metarhizium anisopliae* and *Beauveria bassiana* fungal suspensions on conidial viability, droplet size and deposition coverage in bioassay using a novel bioassay spray system, Biocontrol Sci Techn 23 (2013) 362–366.