Rapid non-destructive method to phenotype stomatal traits

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Abstract

Background: Stomata are tiny pores located on the leaf surface that are central to gas exchange. Stomatal number, size and aperture are key determinants of plant transpiration and photosynthesis, and any variation in these traits can affect plant growth and productivity. Current methods to screen for stomatal phenotypes are tedious, which impedes research on stomatal physiology and hinders efforts to develop resilient crops with optimised stomatal patterning. We developed a rapid non-destructive method to phenotype stomatal traits in four species: wheat, rice, tomato, and Arabidopsis.

Results: The method consists of two steps. The first step is to capture images of a leaf surface directly and non-destructively using a handheld microscope, which only takes a few seconds compared to minutes using other methods. This rapid method also provides higher quality images for automated data analysis. The second step is to analyse stomatal features using a machine-learning model that automatically detects, counts stomata and measures size. The accuracy of the machine-learning model in detecting stomata ranged from 89% to 96%, depending on the species.

Conclusions: We developed a method that combines rapid non-destructive imaging of leaf surfaces with automated image analysis. The method provides accurate data on stomatal features while significantly reducing time for data acquisition. It can be readily used to phenotype stomata in large populations in the field and in controlled environments.

Keywords: Stomata, phenotyping, non-destructive, handheld microscope, machine learning

Background

Stomata are tiny pores located on the surface of leaves and other parts of plants such as the stem and floral parts (1). Stomata play an essential role in gas exchange. About 98% of carbon dioxide (CO2) uptake and water loss from the plant occur through the stomatal aperture (2). When stomata open, it allows plants to uptake CO2 from the atmosphere for producing carbohydrates by photosynthesis. Simultaneously, water evaporates through transpiration (3,4). When water is available, stomata remain open and assimilation and transpiration rates are high, which results in the optimal growth of plants (5). When water is less available, stomata close, which reduces water loss but also reduces CO2 uptake for photosynthetic assimilation, resulting in slower growth. To maintain plant growth and avoid stress, gas exchange must be balanced to maximize CO2 uptake for photosynthesis and minimize the water loss through transpiration (2).

Important factors that influence stomatal transpiration are the stomatal density, and size, the guard cell shape, and the presence or absence of subsidiary cells (4,6). All these factors can affect the amount of CO_2 fixed for photosynthesis, the stomatal conductance, and the water-use efficiency in plants (4). Plants show a range of stomatal sizes and shapes on the leaf epidermis which depends on the plant species, the variety within the species, and the stomatal response to environmental conditions (4).

Considering that the increasing global temperature directly affects drought severity, improving crop water-use efficiency is critical to improving yield under drought conditions (6,7). Because plant water use is controlled by stomata, understanding how stomata develop and respond to the environment will help to identify traits for tolerance to drought. The introduction of optimal stomatal distribution and behaviour into crops will help plants adapt to predicted warmer climates. Current knowledge on stomata is limited because studying and phenotyping stomata is labour-intensive, time-consuming, and costly process. The method

commonly used to investigate stomatal traits is the nail polish method. It consists of applying nail polish on the leaf surface to make an imprint that includes stomata, examining the leaf imprints under a light microscope, and analyse images manually to determine the stomata number and size (8,9,10,11,12). However, this approach has some limitations: the leaf imprint obtained by using nail polish does not consistently provide the desired image, which impedes analysis. One common issue is the unavoidable presence of air bubbles that interferes with the image clarity, which can make data analysis tedious, and thus increase the time required to measure stomatal traits (13). In addition, analysing images manually involves human error and can result in inaccurate data. Another cause of inaccurate data is the irregularity of stomatal imprints, which makes it difficult to focus on all the samples in one image.

Another major limitation in using the nail polish method is the time it takes to acquire data. Up to 20 minutes per sample may be required from nail polish application to image analysis (13), and up to 15 minutes per sample may be required for the manual analysis of the stomata (number and size). This factor limits the number of leaf samples for which phenotyping can be conducted in a day, which prevents the application of the method to be applied on a large scale to screen, such as for measuring stomatal traits in large populations (13).

To overcome this limitation, many methods have included a machine-learning program to accelerate image analysis. However, most of these programs were developed based on images obtained from nail polish imprints (13,14,15). Although machine-learning approach reduces the time taken to acquire data, obtaining satisfactory images using nail polish remains difficult and the method cannot be applied on a large scale. Although some recent methods have used a handheld microscope (HHM) as an alternative for the nail polish

imprints, but these methods either provide information on stomata number or aperture change, not stomata size (14,16,17) and the experimental settings are not portable.

Thus, it is important to develop a rapid non-destructive method for stomata phenotyping that provides information on stomatal features to facilitate research on large populations of stomata in order to characterise the diversity in stomata structure in plants. In this study, we developed a method that rapidly provides information on stomata number and size by combining a HHM for stomata imaging and a machine-learning model to automate stomata analysis of microscope images. We developed this method for four species: wheat, rice, tomato, and Arabidopsis.

Materials and Methods

Plant material

The study focused on four plant species (two monocotyledons and two dicotyledons): wheat (*Triticum.* aestivum cv. Cadenza and Gladius), tomato (*Solanum lycopersicum.* cv. Sweetbite and Mighty Red), rice (*Oryza sativa.* cv. R12), and Arabidopsis (*Arabidopsis thaliana.* cv. Columbia). Wheat, tomato, and rice plants were grown in a glasshouse located at the latitude of 34°58’16.72”S and longitude of 138°38’23.17”E under natural photoperiod. Wheat plants were grown from June to October 2021 with 5 replicates in 20 cm pots containing the UC David soil mix (50% peat and 50% sand), at 22°C/15°C day/night. Tomato plants were grown from July to November 2021 with 10 replicates in 20 cm pots containing UC David soil mix, at 22°C/15°C day/night with fertiliser (All Purpose, Scotts Osmocote, New Zealand). Rice plants were grown from September to December 2021 in 5 replicates in 15 cm pots containing the UC David soil mix, at 29°C/21°C day/night, and were supplemented with an iron solution (EDTA (III) ferric salt, and silwet) for the first 4 weeks. Arabidopsis plants were grown from November 2021 to January 2022 with 12 replicates in 8 cm pots containing

the Arabidopsis mix soil (Coir 3.6L, Perlite 3.6L & Sand 0.25L). Arabidopsis were grown in a growth cabinet at 23°C/19°C day/night, with 12 hours photoperiod. The plants were supplemented weekly with Murashige & Skoog Modified Basal Medium with Gamborg Vitamins (Phyto Technology Laboratories, United States).

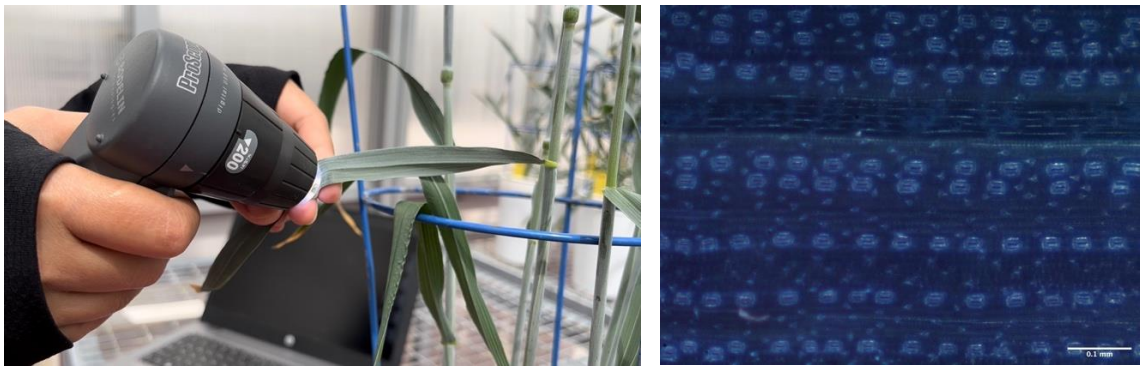
Stomatal imprints: Nail polish method

Stomatal imprints were collected by using nail polish applied to the adaxial and abaxial leaf surface of 4-month-old wheat, 2-month-old rice, 3-month-old tomato and 2-month-old Arabidopsis. Nail polish (Insta-Dri Anti Chip Top Coat, Sally Hansen, USA) was applied gently on the leaf surface in a thin layer, and left to air dry for 5-7 minutes. Clear tape (Crystal clear office tapes, Winc, Australia) was used to peel the dry nail polish off the surface of the leaf and it was put it a microscope slide. A light microscope (Nikon Ni-E compound microscope, Tokyo, Japan) with NIS-elements software (Nikon) was used to capture images of stomatal imprints. The stomatal imprint images were cropped to 0.73 × 0.57 mm for wheat, rice, and tomato and 0.3 × 0.3 mm for Arabidopsis, so that stomatal imprints images and HHM images have the same size to allow for comparison. The number of stomata was determined manually by counting the stomata one by one in each individual image.

Stomata images: Handheld microscope method

An HHM (ProScope, USA) was used to take images of leaves directly **(Fig. 1a)**. The ProScope Capture v6.14 software was used to connect the microscope to a computer. 100×, 200×, and 400× magnifications were used to take images of wheat stomata, while only the 400× magnification was used for rice, tomato, and Arabidopsis, because of the stomata size.

Arabidopsis images taken from the HHM were cropped into 0.3 × 0.3 mm images because the edge of the field of view is generally out of the field of focus.



a **b**

Fig. 1 Collection of stomata images using an HHM. **a.** Image acquisition process. **b.** Wheat stomata image taken by HHM at 200× magnification. Scale bar = 0.1 mm.

Machine-learning model training

Images taken with the HHM were used to train the machine-learning model to recognize stomata separately for each species and magnification. The open-source software LabelImg (18) was used to annotate images by labelling a bounding box around each stomata. The annotated images were uploaded to rowboflow.com for image processing and were made publicly available. The YOLOv5 software was used to train for stomatal detection in Google Colab. The object detection model was saved in Google Drive. A different set of stomata images from the HHM were used to assess the accuracy of the stomata detection model using two parameters: level of precision and recall. Precision measures the model's confidence in classifying a detected object as stomata. Recall reflects the percentage of detected stomata among all existing stomata (19). In the model, stomata number was determined by the number of bounding boxes around stomata, and stomata size refers to the area of the bounding box.

Results

Optimal magnification

The HHM has three different magnifications: 100× (2.87 × 2.17 mm field of view); 200× (1.36 × 1.03 mm field of view); and 400× (0.75 × 0.57 mm field of view). Each magnification could provide information on the stomata number and/or size, depending on the plant species (Supp. Fig. 1). For wheat, a 100× magnification could be used to count the number of stomata (Supp. Fig. 1a), whereas the 200× magnification was suitable for both counting the number and determining the size of stomata (Supp. Fig. 1b). The 400× magnification could also be used for determining the number and size of wheat stomata (Supp. Fig. 1c). For rice, tomato, and Arabidopsis, only the 400× magnification could be used to determine the number and size of stomata (Supp**.** Fig**.** 1 d-f) because their stomata are smaller than those observed in wheat.

Comparison between nail polish images and HHM images

The number of stomata counted in nail polish images and HHM images were comparable in wheat, tomato and Arabidopsis, but the number of stomata counted in HHM images were slightly higher than in nail polish images in rice **(Table 1)**.

Table 1 Comparison between the number of stomata obtained from nail polish imprints and HHM images.

Name of plants	Number of Stomata	
	Nail polish imprint	HHM images
Wheat	18–35	22–37
Tomato	51–71	60–82
Arabidopsis	14–21	15–25
Rice	77–110	115–151

The HHM provided better quality images faster than did the nail polish method. Although images taken using the light microscope from nail polish imprints were at higher

173 resolution, air bubbles were frequently present in the samples **(Fig. 2 a, c, e, g)**. Images taken
174 with the HHM were at a lower resolution but were clear enough to allow for automated
175 recognition and analysis using the machine-learning model **(Fig. 2 b, d, f, h)**.
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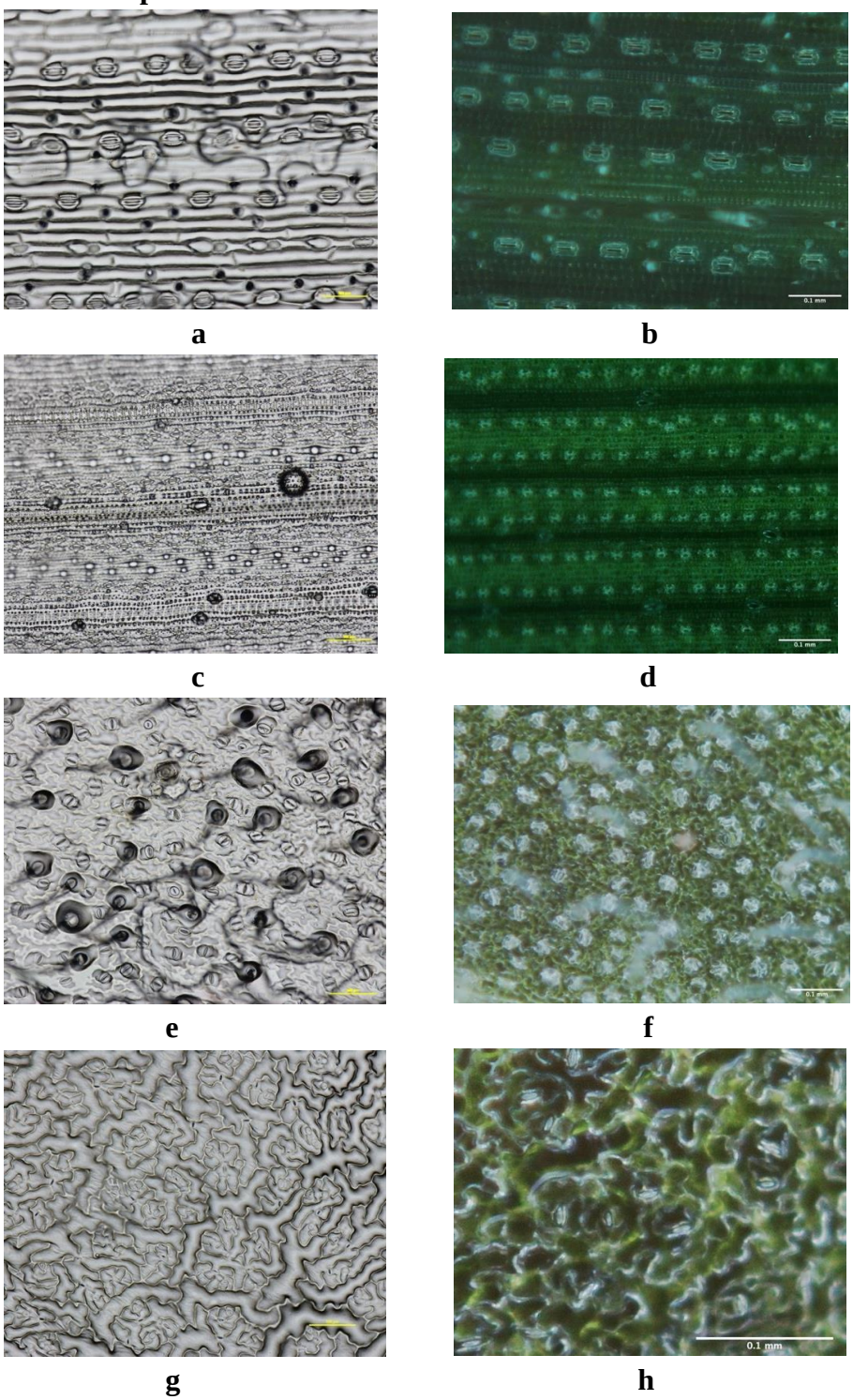

Fig. 2 Images from nail polish imprints vs handheld microscope on wheat (a, b), rice (c, d), tomato (e, f), and Arabidopsis (g, h). Scale bar = 100 µm

Images of wheat leaves using 100× magnification were not at high resolution, but stomata were still recognizable using the machine-learning program. Images at 200× and 400× magnifications were clearer, which allowed to measure both the number and the size of stomata. For rice, the nail polish imprints were uneven, which not allow to focus on all stomata in one image; hence, at least two images at a different focus per sample needed to be taken. Given their smaller size, the stomata in rice leaves could only be observed using the 400× magnification of the HHM. The images obtained were a little blurry, but stomata could still be recognized and analysed using the machine-learning program. For tomato, the nail polish imprints were as clear as the images taken with the HHM and stomata could be seen with a 400× magnification. In contrast, for Arabidopsis, the nail polish images were clearer and provided more accurate results than did the HHM images. Even with the 400× magnification, the HHM could only take images with a limited focus area on the abaxial leaf surface. Images taken of the adaxial leaf surface were blurry and did not allow for automated stomata recognition and analysis. However, the HHM significantly accelerated image acquisition (Fig. 3).

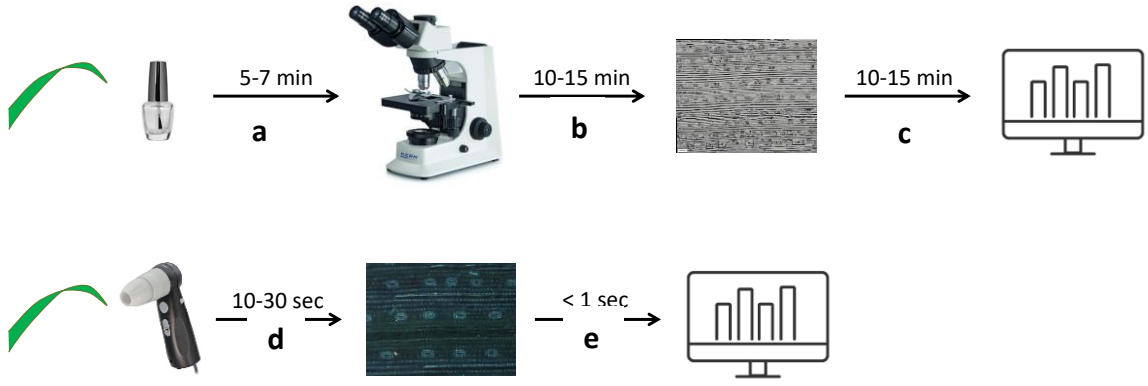

Fig. 3 Nail polish method vs rapid method. **a** Nail polish Imprint. **b** Light microscope imaging. **c** Manual analysis. **d** Handheld microscope imaging. **e** Automated analysis.

193 **Accuracy of the machine-learning model**

194 The precision of the wheat model was best under the 400× magnifications compared to the
195 200× and 100× (0.99, 0.91 and 0.954, respectively – Table 2). Wheat had the best precision
196 compared to rice, tomato and Arabidopsis using the same 400× magnification (0.99, 0.89,
197 0.84 and 0.74, respectively). The recall was high for wheat (0.99), rice (0.92), and tomato
198 (0.91) but was lower in Arabidopsis (0.77) (Table 2, Fig. 4).

199

200 **Table 2 Statistical result of the model**

Stomata detection model	Precision	Recall
Wheat (400×)	0.99	0.997
Wheat (200×)	0.91	0.91
Wheat (100×)	0.954	0.978
Rice	0.888	0.916
Tomato	0.843	0.911
Arabidopsis	0.741	0.769

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202

203 **Accuracy of the results using the machine-learning program**

204 Stomata detection using the machine-learning model was highly accurate, meaning that the
205 model was able to detect most of the stomata in an image (Table 3). In wheat, accuracy for
206 the 100x, 200x and 400x magnifications was 89%, 95.8% and 95.6%, respectively. The
207 accuracy of the model was 92.3% for rice, 88% for tomato and 89.5% for Arabidopsis with
208 the 400x magnification (Fig. 4, Table 3).

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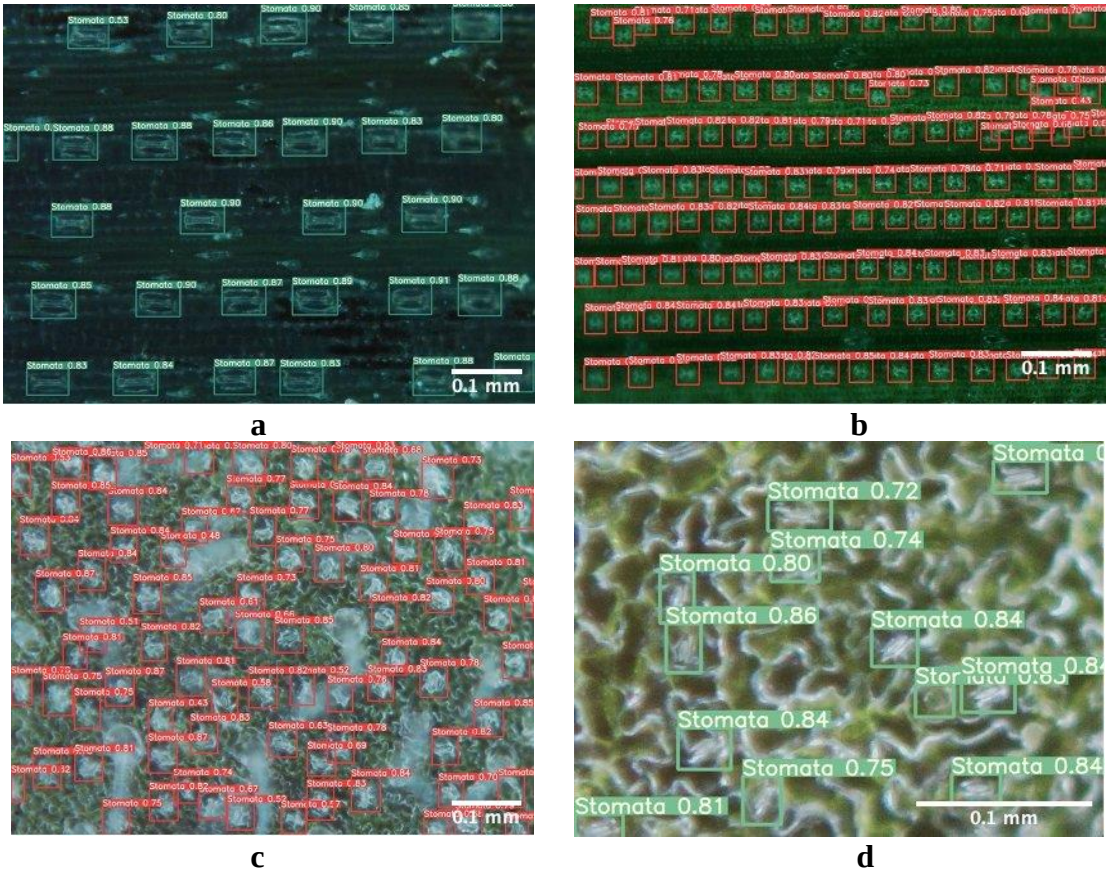

Fig. 4 Stomata detection using the machine-learning model in wheat (a), rice (b), tomato (c) and Arabidopsis (d) images using the 400x magnification. The model detects and labels stomata with bounding boxes and gives the confidence number in each box. Scale bar = 0.1 mm.

Table 3. Average percentage of total stomata detected by the models in each species.

Species	Accuracy (%)
Wheat 400×	95.56
Wheat 200×	95.79
Wheat 100×	89.15
Rice 400×	92.25
Tomato 400×	87.96
Arabidopsis 400×	89.49

Discussion

The HHM provided clear images of the wheat, rice, tomato leaf surface, and Arabidopsis adaxial leaf surface non-destructively in a few seconds. Image capture using the HHM depends on experience and environmental conditions. The optimal conditions for taking stomata images using a HHM are when the sky is cloudy because it reduces the sunlight intensity that can influence image clarity and can interfere with viewing the image on the screen before taking it. If sunlight is unavoidable, the experimental setting can be shaded to minimize sunlight interference.

The advantages of this method are the speed of taking clear images by using the HHM and the automated analysis of number and size of stomata that is highly accurate. The HHM can be used to take images anywhere as it is portable. The images taken can be viewed immediately and other images of the same leaf can be taken if one image is not satisfactory. Once all leaf images are taken, it can be directly uploaded to the stomata detection model and the results can be obtained within seconds. In contrast, the nail polish method requires more time to obtain leaf imprints, transport of samples to the light microscope and more to view and capture the images by using a light microscope, then analysing stomata manually which takes up to 30 minutes per sample. If the images are not satisfactory after this long process, other leaf imprints need to be taken, which is also time consuming. Although some recent methods included a machine-learning program to analyse stomata automatically, they still require taking leaf imprints using nail polish and taking images using a light microscope (8,14,15).

In this experiment, it was difficult to take clear images of the adaxial side of Arabidopsis leaves. The difficulties encountered might be due to the different structure of the adaxial leaf surface where mesophyll cells are more densely packed compared to the abaxial surface where there is space between cells (20).

A high throughput (30 seconds per image) stomatal phenotyping method opens up the opportunities for screening large populations for variation in stomatal traits. The was not previously achievable due to the length of time it took to obtain images with the nail polish technique. Using this method, stomatal traits can be screened for in large populations such as biparental mapping and genome-wide association (GWAS) panels, which require a large number of populations to be genotypic and phenotypic (21,22). Many studies on drought, heat and salt stress have been looking at biparental mapping and GWAS populations to investigate the association between genotype and phenotype by screening for morphological traits such as biomass or plant weight (22,23,24,25,26,27). Stomatal traits can now be screened in such populations using the proposed method.

In the future, the proposed method can be developed further by including measurements of stomatal aperture in the model analysis, so the users can have more information available to improve their decision-making. The method could also be adapted to identify other leaf traits such as trichome and plant epidermal cells as the HHM can capture those features that can be detected automatically using machine-learning.

This rapid method is affordable and can be readily used by any individual since it does not require specific skills in computer science or programming. The user needs to take images using an HHM and download images on the image analysis pipeline available on Google Colab (Supp. 3).

Conclusion

The proposed method provides a rapid non-destructive tool for stomata phenotyping, that is, determining stomata number and size. The experimental setup is portable and allows stomata phenotyping at a large scale, which will allow breeders to accelerate identification of new

traits for drought tolerance in crops. Further, the model developed can be trained with images of other species using the same pipeline.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

AE and SR conceived the project. AE, SR and PP designed the experiments. PP and AE performed the experiments. PP, ZZ and AE analysed the data. PP, ZZ, SR and AE interpreted the data. All authors drafted the manuscript.

References

1. Willmer C, Fricker, M. The Distribution of Stomata. In: Black, M. and Charlwood, B. Stomata. 2nd. Netherlands: Dordrecht, Springer; 1996. P. 12-35.

2. Lawson T, Blatt MR. Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency. Plant Physiol. 2014;164(4):1556-70.

3. Nunes TDG, Zhang D, Raissig MT. Form, development and function of grass stomata. Plant J. 2020;101(4):780-99.

4. Bertolino LT, Caine RS, Gray JE. Impact of stomatal density and morphology on water-use efficiency in a changing world. Front Plant Sci. 2019;10:225.

5. Caine RS, Yin X, Sloan J, Harrison EL, Mohammed U, Fulton T, et al. Rice with reduced stomatal density conserves water and has improved drought tolerance under future climate conditions. New Phytol. 2019;221(1):371-84.

6. Harrison EL, Arce Cubas L, Gray JE, Hepworth C. The influence of stomatal morphology and distribution on photosynthetic gas exchange. The Plant journal: for cel and molecular biology. 2020;101(4):768-79.

7. Hatfield JL, Dold C. Water-Use Efficiency: Advances and Challenges in a Changing Climate. Frontiers in Plant Science. 2019;10.

8. Scarpeci TE, Zanor MI, Valle EM. Estimation of stomatal aperture in Arabidopsis thaliana using silicone rubber imprints. Bio Protoc. 2017;7(12):e2347-e.

9. Berger D, Altmann T. A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in Arabidopsis thaliana. Genes Dev. 2000;14(9):1119–31.

10. Scarpeci TE, Frea VS, Zanor MI, Valle EM. Overexpression of AtERF019 delays plant growth and senescence, and improves drought tolerance in Arabidopsis. J Exp Bot. 2017;68(3):673–85.

304 11. Kagan ML, Novoplansky N, Sachs T. Variable cell lineages form the Functional pea
305 epidermis. Ann Bot. 1992;69(4):303–12.
306 12. Schlüter U, Muschak M, Berger D, Altmann T. Photosynthetic performance of an
307 Arabidopsis mutant with elevated stomatal density (sdd1-1) under different light regimes. J
308 Exp Bot.
309 13. Millstead L, Jayakody H, Patel H, Kaura V, Petrie PR, Tomasetig F, et al.
310 Accelerating automated stomata analysis through simplified sample collection and imaging
311 techniques. Front Plant Sci. 2020;11(1493).
312 14. Jayakody H, Liu S, Whitty M, Petrie P. Microscope image based fully automated
313 stomata detection and pore measurement method for grapevines. Plant Methods.
314 2017;13(1):94.
315 15. Kwong QB, Wong YC, Lee PL, Sahaini MS, Kon YT, Kulaveerasingam H, et al.
316 Automated stomata detection in oil palm with convolutional neural network. Scientific
317 Reports. 2021;11(1):15210.
318 16 Liang X, Xu X, Wang Z, He L, Zhang K, Liang B, et al. StomataScorer: a portable
319 and high-throughput leaf stomata trait scorer combined with deep learning and an improved
320 CV model. Plant biotechnology journal. 2022;20(3):577-91.
321 17. Zhuangzhuang S, Yunlin S, Qing L, Jian C, Xiao W, Qin Z, et al. An Integrated
322 Method for Tracking and Monitoring Stomata Dynamics from Microscope Videos. Plant
323 phenomics. 2021;2021:9835961-.
324 18. Tzutalin. LabelImg. Git code. 2015. https://github.com/tzutalin/labelImg
325 19. Powers DMW. Evaluation: from precision, recall and F-measure to ROC,
326 informedness, markedness and correlation. 2020.
327 20. Nakata M. and Okada K. The Leaf Adaxial-Abaxial Boundary and Lamina Growth.
328 Plants 2013, 2(2), 174-202.

329 21. Flint J. GWAS. Current biology. 2013;23(7):R265-R6.

330 22. Jabbari M, Fakheri BA, Aghnoum R, Mahdi Nezhad N, Ataei R. GWAS analysis in

331 spring barley (Hordeum vulgare L.) for morphological traits exposed to drought. PloS one.

332 2018;13(9):e0204952-e.

333 23. Abdelraheem A, Thyssen GN, Fang DD, Jenkins JN, McCarty JC, Wedegaertner T, et

334 al. GWAS reveals consistent QTL for drought and salt tolerance in a MAGIC population of

335 550 lines derived from intermating of 11 Upland cotton (Gossypium hirsutum) parents.

336 Molecular genetics and genomics: MGG. 2020;296(1):119-29.

337 24. Li B, Chen L, Sun W, Wu D, Wang M, Yu Y, et al. Phenomics-based GWAS analysis

338 reveals the genetic architecture for drought resistance in cotton. Plant biotechnology journal.

339 2020;18(12):2533-44.

340 25. Prado SA, Cabrera-Bosquet L, Grau A, Coupel-Ledru A, Millet EJ, Welcker C, et al.

341 Phenomics allows identification of genomic regions affecting maize stomatal conductance

342 with conditional effects of water deficit and evaporative demand. Plant, cell and environment.

343 2018;41(2):314-26.

344 26. Scott MF, Ladejobi O, Amer S, Bentley AR, Biernaskie J, Boden SA, et al. Multi-

345 parent populations in crops: a toolbox integrating genomics and genetic mapping with

346 breeding. Heredity. 2020;125(6):396-416.

347 27. Furbank RT, Tester M. Phenomics – technologies to relieve the phenotyping

348 bottleneck. Trends in Plant Science. 2011;16(12):635-44.