

Aspect and Rootstock Genotype Influence Leaf Stomatal Density in *Macadamia* sp. †

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Abstract: We investigated the effects of three rootstocks (Beaumont, Daddow and WJMAS-29), and two aspects (north and south), on leaf stomatal density (SD) of a common macadamia scion ‘HAES741’. Manual counting and artificial intelligence (AI) image processing methods were used to measure SD from the leaves of the scion grafted onto each rootstock. In both methods, rootstock and aspect showed significant effects on scion SD (352 stomata mm⁻² in WJMAS-29, 310 stomata mm⁻² in Beaumont, 332 stomata mm⁻² in Daddow; 358 stomata mm⁻² in north and 305 stomata mm⁻² in south). We discussed that variability in SD due to rootstock and aspect can be used to improve productivity of future orchards systems.

Keywords: macadamia; rootstock; stomata; aspect; image processing; AI; disease susceptibility; climate change

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1. Introduction

Stomata, the pores on the leaf epidermis, facilitate gas exchange in plants. Plants balance their CO₂ demands and water losses through adjustment of stomatal size, density and modulation (opening and closing of stomata) [1,2]. Plants are vexed with the competing priorities of CO₂ acquisition and water conservation. Opening stomata increases CO₂ concentration within the plant for photosynthesis, however, this results in water losses through transpiration [3]. Adjustment of stomatal characteristics may therefore have significant impacts on photosynthetic performance, disease susceptibility, water use efficiency and climate change adaptability [4,5]. A wide array of genetic and environmental factors exert control over the stomatal phenotype [6,7]. To the authors knowledge, there has not been any research undertaken to evaluate the stomatal characteristics of macadamia under different environments, nor into investigating potential rootstock effects on the scion. As macadamia is a vegetatively propagated plant, rootstock effects on plant productivity should be an important consideration in the breeding program. In addition to this, the effects of shading on stomatal density are of interest. There is ample evidence to suggest that stomatal density is affected by aspect, however, so far as we know, no investigation has yet been undertaken in macadamia [8,9]. Optimal parameters for stomatal characteristics for sustainable and productive macadamia orchards remain unknown. Using manual counting and an AI-based image processing method, we determined the effects of aspect and rootstock genotype on leaf stomatal density of a common scion. We also discussed the usefulness of our findings for future orchard management decisions for sustainability and climate change adaptation.

2. Materials and Methods

2.1. Plant Material and Experimental Design

This experiment utilised an existing macadamia rootstock trial, which was planted in 2017 at the Queensland Department of Agriculture and Fisheries Maroochy Research Facility [10]. In this study, three cutting-grown rootstocks (three replications each) grafted to the common scion 'HAES741', were chosen to investigate the effects of rootstock and aspect on leaf stomatal density: Daddow, Beaumont and WJMAS-29.

2.2. Phenotyping for Stomatal Density

The protocol outlined below was adapted from the lab guide issued by the Gene Technology Access Centre [11]. This method uses nail polish to create a negative imprint of the leaf surface, which is later mounted to a microscope slide for imaging. Sampling was conducted within a two-hour time period; each tree was located, and branches were selected from north and south aspects. A 1 × 3 cm approx. strip of nail polish was spread onto the abaxial leaf surface, avoiding the midrib, of the first three fully expanded mature leaves on the branch and was left to dry. All trees were treated before returning to the first tree to commence the bagging of samples. Each painted leaf was cut from the branch and placed in a labelled bag. Samples were then taken to the lab to commence slide mounting.

In the lab, the dried nail polish was removed from the surface of the leaf using a 10cm length of sticky tape placed over the entire nail polish negative. Once peeled from the leaf, this negative and sticky tape composite was placed sticky side down onto the glass microscope slide, smoothed to reduce air bubbles, trimmed with a scalpel blade and labelled. Each slide was then photographed five times at 400× magnification using a compound microscope equipped with a camera adapter. The locations of the five photographs taken for each sample were randomly selected by moving the microscope stage to a unique position and capturing a photograph. The field of view was calculated by using a calibration slide to measure length and width of the area in question. At 400× magnification, the photograph represents 0.053 mm² (0.28 mm × 0.19 mm). Photograph file names were recorded with the respective sample and sub sample numbers in an Excel spreadsheet.

2.3. Stomatal Quantification

Two methods were used to count the stomata: manual counting (M), and an artificial intelligence (AI)-based image processing tool. With the manual method, the protocol was to count only stomata which had both guard cells fully within the boundaries of the picture. The AI method utilised a beta web application [12,13]. Once all counts were recorded, stomatal density was calculated using the stomatal count and the known area to obtain a stomatal density presented as number of stomata mm⁻².

2.4. Statistical Analysis

Analysis of the data was completed in Genstat [14]. Restricted maximum likelihood (REML) mixed model analysis was conducted to identify the significant effects of the factors and to determine the variability in leaf stomata density of the common scion 'HAES741'.

3. Results and Discussion

3.1. Rootstock Affects Stomatal Density

REML mixed model analysis showed that stomatal density differed significantly with respect to rootstock choice ($p \leq 0.001$ (manual count), $p = 0.003$ (AI count)) (Table 1).

Table 1. Restricted maximum likelihood (REML) mixed model analysis output.

Fixed Term	df	Wald Statistic		F Probability	
		Manual	AI Count	Manual	AI Count
Rootstock	2	37.71	11.69	<0.001	0.003
Aspect	1	93.44	60.94	<0.001	<0.001
Rootstock × Aspect	2	1.58	0.48	0.455	0.788
Rootstock × Replicates	6	50.16	28.97	<0.001	<0.001
Rootstock × Leaf × Sample	6	9.09	1.02	0.174	0.985
Rootstock × Leaf × Sample × Image	36	15.2	18.64	0.999	0.99
Aspect × Leaf × Sample	2	0.1	0.38	0.949	0.825

In this study, rootstock effect on leaf stomatal density of ‘HAES741’ varied from 310 mm⁻² to 352 mm⁻², with the highest SD due to the effect of ‘WJMAS-29’, and lowest SD due to the effect of Beaumont (Figure 1). These results indicate that rootstock choice does in fact play a role in the expression of the stomatal phenotype. This knowledge has important implications for the rootstock breeding program, as stomatal characteristics are likely to affect the water use efficiency, photosynthetic performance and disease susceptibility of macadamia [4,15–17]. A 2018 study on the stomatal characteristics of *Arabidopsis thaliana*, found that stomatal size and density are negatively correlated ($r = -0.5$, $p < 0.001$) [4]. This is of interest, as it was also found that stomatal size correlates with water use efficiency, with smaller stomata responsible for higher water use efficiency [4,18]. So far, no investigation into macadamia has been undertaken to explore the relationship of stomatal characteristics with respect to water use efficiency. It has been proposed that smaller stomata are able to react more quickly to environmental stimulus due to their inherently larger surface area to volume ratios, allowing faster ion flow through the guard cell membrane; the dynamic cells of the stomatal complex responsible for the opening and closing of the stomatal aperture [2]. This would allow the plant to fine tune stomatal apertures to optimise stomatal conductance in accordance with current environmental conditions, thereby minimising evaporative losses and maximising CO₂ acquisition. For the first time in macadamia, we identified that rootstocks affect stomatal characteristics, which can be used to select rootstocks to maximise water use efficiency and CO₂ acquisition. As this study used only three rootstocks, and was conducted in a single year, further investigation is necessary to confirm repeatability of this result. We also suggest exploring the relationships of stomatal characteristics on water use efficiency and gas exchange in macadamia.

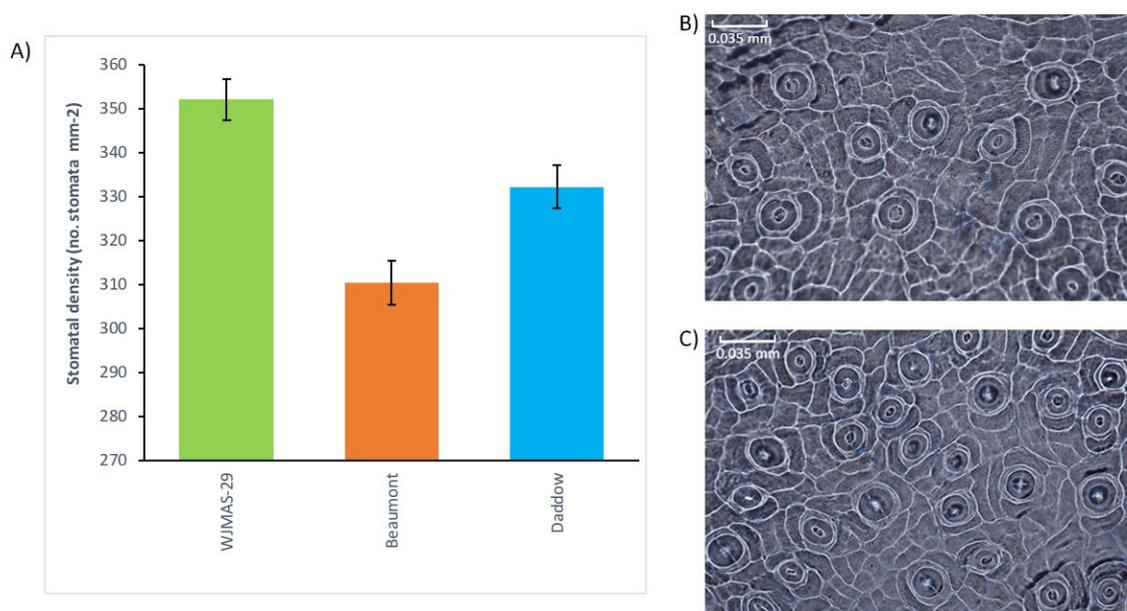


Figure 1. Effect of rootstocks on leaf stomata density of HAES741. Error bars show the standard error (SE) of each mean. (A) Variability in stomata density due to three rootstocks. (B) Low stomatal density due to the effect of Beaumont. (C) High stomatal density due to the effect of WJMAS-29.

One potential downfall of increased stomatal density is the possibility of increased incidence of husk spot disease, caused by *Pseudocercospora macadamiae*. *P. macadamiae* is an endemic fungal pathogen in Australian macadamia production systems [16]. It germinates on the surface of the fruit husk, where it elongates and enters through open stomata and subsequently proliferates through the pericarp tissue. It is unknown whether leaf stomatal density is correlated to husk stomatal density, however, if true, more open pores on the husk surface could result in increased susceptibility to husk spot disease.

3.2. Aspect Affects Stomatal Density

Our investigation on the north and south sides of plants identified that stomatal density differed significantly with respect to aspect ($p \leq 0.001$ (manual count), $p \leq 0.001$ (AI count) (Table 1). Leaf stomatal density on the northern aspect had a mean of 358 stomata mm⁻², which was significantly higher than that of leaves on the southern aspect (305 stomata mm⁻²) (Figure 2). Leaf stomatal density was significantly higher on the northern aspect for all rootstocks; WJMAS-29—north 381 stomata mm⁻², WJMAS-29—south 315 stomata mm⁻², Beaumont—north 327 stomata mm⁻², Beaumont—south 281 stomata mm⁻², Daddow—north 348 stomata mm⁻², Daddow—south 300 stomata mm⁻². This data demonstrates there was no interaction between aspect and rootstock ($p = 0.455$ (M), $p = 0.788$ (AI)) (Table 1). A possible explanation for this effect is that due to reduced light interception received on the southern aspect (in southern hemisphere production systems), there is a reduced need for gas exchange due to reduced photosynthetic rate [5]. Jumrani et al. [6] demonstrated a similar relationship in soybean, where shade cloth of increasing impermeability resulted in decreased stomatal density, as well as decreased yields. In addition to this, lower temperatures would also reduce the localised vapour pressure deficit at the leaf surface, so transpirational losses would be less severe. This highlights the importance of designing efficient orchards, and utilising small trees to minimise interrow shading and maximise solar gain [19]. This information also has important implications for future studies. Knowledge of within-tree heterogeneity, with respect to stomatal density, will reduce the chances of confounding genetic effects with those of environmental origin. Our results showing differences between sun and shade aspects match those of similar studies on other tree species [8,9].

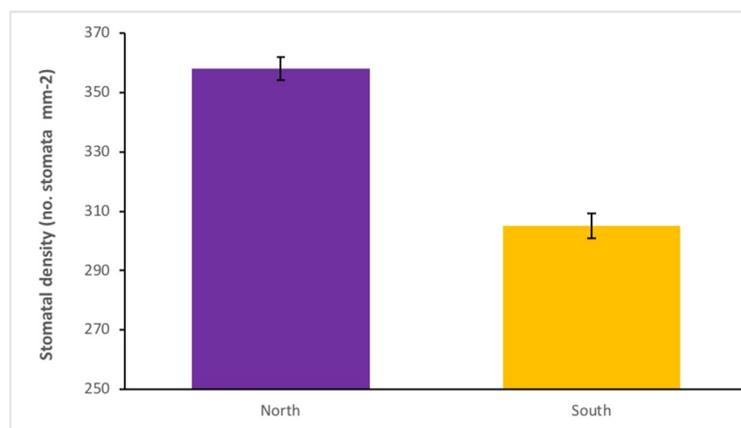


Figure 2. Mean leaf stomatal density of three macadamia rootstocks on northern aspect (purple) and southern aspect (yellow), using HAES741 as a common scion. Error bars show the standard error (SE) of each mean.

3.3. Comparison of Manual and Artificial Intelligence Quantification Methods

Stomatal counts were carried out using manual and AI methods, with the aim of establishing whether the AI method was of comparable accuracy to manual counting. We ran a correlation on the manual and AI data sets to assess the suitability of the technology for further studies. The results indicate there is a strong correlation between the two methods ($R^2 = 0.7018$) (Figure 3).

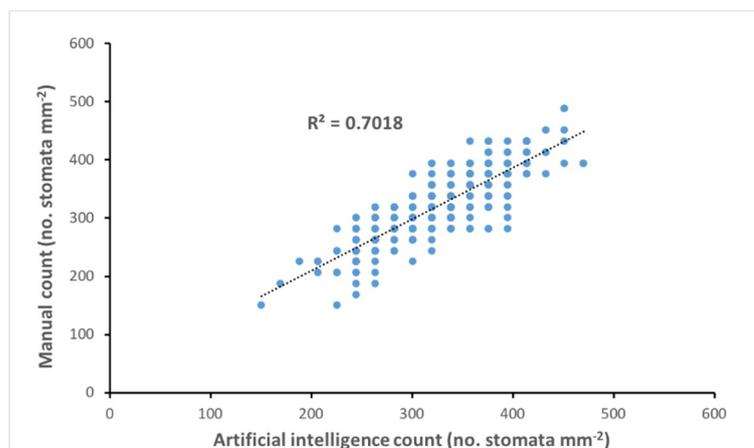


Figure 3. Correlation of artificial intelligence (AI) vs. manual counting methods for determining stomatal density.

One of the limitations of the AI method was the differences in counting protocol. The manual method did not count stomata that were half in/half out of the microscope field of vision, whereas the AI method was able to identify the outline of some of these stomata on the borders of the frame and counted them. Therefore, correlation may in fact be higher than reported if protocols were more closely aligned. Additionally, some stomata were not picked up by the AI software, which may be due to photo resolution or visual artifacts from the slide mounting process.

It was initially anticipated that the AI method would offer time savings over manual counting, which would help with the efficiency of phenotyping. However, due to the process requiring individual upload of each image, and allowances for AI processing, time savings were not deemed to be substantial enough to warrant use of this tool in its current form. The utility of this tool could be greatly increased by, (a) allowing batch processing of images, and (b) production of a data file with automated stomatal density calculations.

This would drastically reduce the amount of time required to phenotype samples, which may be useful for more extensive studies.

4. Conclusions

This preliminary research investigated the effects of rootstock and aspect on stomatal density of macadamia. We found that rootstock choice affects stomatal density, which has potential implications on water use efficiency, photosynthetic performance and disease susceptibility of macadamia. Additionally, we found that aspect also affects stomatal density, with leaves on the northern side of the canopy (southern hemisphere) having a higher stomatal density than those on the southern side. This result highlights the importance of canopy management for orchard productivity. Limitations of the study include small rootstock and scion genotype sample sizes, and slight differences in stomatal counting protocol between the manual and AI quantification methods. Further investigation of stomatal characteristics in macadamia is required, especially the relationship between stomatal size and density.

Author Contributions: S.W. conducted experimentation, B.T. and M.A. supervised the project, all authors have read and agreed to the published version of the manuscript.

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