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Leaf Stomatal Density and Distribution in Black Pepper under Field Conditions

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ABSTRACT

Currently, there is little information on whether black pepper leaf has more stomata on the underside, above side or the stomata are equally distributed. Thus, this paper provides vital information on black pepper leaf stomata distribution and density to shed more light on the subject. The objective of this study was to investigate the distribution pattern of stomata on developing and matured leaf of *Piper nigrum* var. Kuching, Semongok Emas and Semongok Aman. To analyse the leaves, we used nail polish staining tools combined with a compound microscope incorporated with imaging software. Our analysis indicated that stomata occur only on the lower side of the leaf (hypostomatic). The result on distribution showed that except for matured leaf of the Kuching variety, abaxial stomata were found abundantly at the base of the leaf in all varieties and developmental stages. Stomatal densities according to varieties in *P. nigrum* leaves recorded that the Semongok Aman has the highest stomata count followed by Semongok Emas and Kuching. Differences in stomatal density between leaves of varying developmental stages showed that developing leaves of *P. nigrum* have more stomata count than the matured leaves. Further analysis with the LI-6400XT Portable Photosynthesis System revealed that photosynthetic, stomatal conductance and transpiration rates on the underside (abaxial) of the leaf were significantly higher than on both sides. Information obtained from this study may contribute significantly in subsequent research such as foliar fertiliser application or gas exchange measurements.

Keywords: Stomatal density, stomata distribution, gas exchange rate, *Piper nigrum*

INTRODUCTION

Stomata are structures found in the above ground parts of all terrestrial plants and account for approximately 95% of gas exchange (Martins et al., 2012). They are epidermal valves that control the plant carbon dioxide input and water output, directly influencing carbon assimilation, plant water status and water use efficiency (Bergmann and Sack, 2007). These structures are therefore key components for the survival of terrestrial plants (Frank et al., 2017) and play a major role in the response of plants to environmental stress (Sulok et al., 2016). During the development of the leaves, the stomata are formed by the simultaneous processes of asymmetric cell division and cell differentiation giving rise to the stomatal density (SD) and the spatial distribution of stomata on the leaf surface is genetically regulated during this period (Bergmann and Sack, 2007). In addition, irrespective of plant age, the ontogeny of stomata is also modulated by environmental factors that may change through time, such as the intensity and quality of radiation, humidity, temperature, carbon dioxide and ozone levels in the atmosphere, soil moisture and nutritional availability. Based on the leaf position in a plant, the part of the leaf which is more exposed to sunlight irradiance, humidity and carbon dioxide usually demonstrates higher stomata distribution and density due to the need to conduct gas exchange processes such as photosynthesis and transpiration (Martins et al., 2011).

The heterogeneity of the spatial distribution of stomata on the leaf epidermis caused by genetic and environmental factors is a characteristic of plants that has been known for some time (Ravindram et al., 2005). Currently, there is little information on whether black pepper leaf has more stomata on the underside, above side or the stomata are equally distributed. There have been very few published studies that provide detailed information about the number, size and location of stomata on the pepper leaf surface. Without this knowledge, foliar fertiliser application will be less effective if the targeted foliar spraying area is not studied properly. This can lead to wastage, run-off and even environmental pollution if the foliar fertilisers are not fully utilised. Furthermore, when determining gas exchange performance particularly on net photosynthesis, transpiration and stomatal conductance rate, it is important to make sure that the equipment used is set properly based on the stomata density and distribution for better accuracy (LICOR, 2011).

Thus, this paper will provide vital information on leaf stomata distribution and density of black pepper for future research. The objective of this study was to investigate the spatial distribution pattern of stomata on leaf surfaces. This information will be useful for research that is associated with gas exchange performance study.

MATERIALS AND METHODS

The experiment was conducted in a black pepper farm located in Kampung Karu, Kota Padawan, Sarawak, Malaysia at longitude and latitude of 01^{o} 20' N and 110^{o} 1' E. The terrain of the 0.2 hectare plot was relatively flat. The climate is tropical, moderately hot with average annual rainfall around 3500 mm to 4000 mm. The region is directly exposed to the northeast monsoon with 50% to 60% of the rainfall occuring during the monsoon season from November to February. The dry season is from April to September, with June and July as the driest months of the year. The temperature in Kota Padawan ranges from 20 to 36 °C with average temperature around 23 °C in the early hours of the morning and rises to around 32 °C in the midafternoon (Sarawak Tourism Board, 2008). The soil at the experimental site was previously surveyed by the Department of Agriculture, Soil Branch, Kuching, Sarawak and was named as Sedong series which is a fine, mixed, isohyperthermic, Tipik Distroparadanks (Soil Survey Staff, 1992; Paramananthan, 2000). Three Malaysian Pepper Board (MPB) recommended pepper varieties (Figure 1) namely *P. nigr*um var. Kuching, *P. nigrum* var. Semongok Aman and *P. nigrum* var. Semongok Emas under field conditions were randomly selected and assessed in this research. All three varieties are recommended based on selected characteristics including high yielding, tolerant to certain pests and diseases besides their ability to live longer than other varieties (Lai et al., 2017). Approximately 10 pepper vines per variety involved in this study were aged 3 years and above. Standard farming practices recommended by the MPB were used in this study (Lai et al., 2017). The samples used were based on developmental stages of the leaf which were the developing and mature stages. Developing leaf refers to leaf which is still undergoing the process of maturation whereas mature leaf refers to leaf that has acquired the final leaf shape and size but before the senescence stage. The treatments were developing leaf of the Kuching variety (KCH1), developing leaf of the Semongok Emas variety (SE1), developing leaf of the Semongok Aman variety (SA1), mature leaf of the Kuching variety (KCH2), mature leaf of the Semongok Emas variety (SE2) and mature leaf of the Semongok Aman variety (SA2). All treatments were replicated 12 times, giving a total of 72 leaves altogether being assessed in this study. Duration of the study was from January 2018 to July 2018.

Stomata analysis

Obtaining stomata impressions were the preliminary work for stomata analysis. A thick layer of transparent nail polish was painted evenly on the adaxial and abaxial surfaces at four different parts of each leaf which were the tip, the middle, the side and the base (Figure 2). After 10 minutes, the layer of dried nail polish was peeled gently from the leaf. The layer was then placed on a very clean slide and then covered with a glass slide cover. The slide was then viewed under a compound microscope (DM4000B, LEICA, Wetzlar, Germany) installed with imaging software to make a count for estimating the number of stomata on both leaf surfaces. The stomata were observed under 40 X magnification with an image size of 0.515 mm x 0.386 mm. A representative section of stomata density was chosen and the stomata densities were calculated. Stomata densities were analysed according to specific area distribution, varieties and developmental stages of the *P. nigrum* leaves.

Gas exchange measurement

The measurements on the gas exchange including photosynthetic rate, transpiration rate and stomatal conductance rate were also determined in this study (Mehar-un-Nisa et al., 2017). These measurements were done using an infrared gas analyser (LI-6400XT, LI-COR, Lincoln, USA). Stomata ratio was set at “1” for equal stomata density on top and bottom of leaf whereas “0” for stomata on one side only to observed differences in the gas exchange rates (LICOR, 2011). Measurements of net photosynthesis on an area basis (A) (µmol $CO_2/m^2/s^1$), leaf stomatal conductance (gs) (mol $H_2O/m^2/s^1$) and transpiration rate (E) (mmol $H_2O/m^2/s$) of 12 different leaves per variety were monitored. Light intensity (Photosynthetically Active Radiation, PAR) within the sampling chamber was set to PAR at 1500 µmol/m^2/s which was the intensity where photosynthetic rates for black pepper would be maximal. The CO_2 flow into the chamber was maintained at a concentration of 400 µmol/mol. The humidity flow into the chamber was fixed at 500 µmol/s. Measurement was done on gas exchange parameters at between 1100 to 1200 h which was the period where gas exchanges were optimum.

Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA) with the SPSS software (version 15). Data was analysed statistically by Tukey’s Honest Significance Difference (HSD) Test, at $\alpha = 0.05$ level of significance and independent t-test to detect treatment effect.

RESULTS AND DISCUSSION

A study by Ravindram et al. (2005) noted that stomata in black pepper occur only on the lower or abaxial side of the leaf. This type of stomata distribution is known as hypostomatic. The type of stomata in black pepper is shown in Figure 3. The stomata are generally anisocytic or unequal in size and surrounded by two guard cells as well as four rings of subsidiary cells. In a similar observation, Ravindram et al. (2005) described the stomata formation as “mixed type” where new meristemoids appeared between older ones or between mature stomata. In this mesoperigenous development, the meristemoid divides unequally to form a large and a small cell. The latter shows a prominent nucleus and darker staining cytoplasm and it divides once again by a wall placed at right angles to the first to form two smaller cells which are more or less rectangular. One of these cells becomes the guard cell mother cell and the two other mesogene cells formed by the above divisions become the neighbouring cells flanking the two sides of the guard cell mother cell.

Figure 1. Developing and matured black pepper leaves from recommended varieties. The leaves were developing leaf of the Kuching variety (a), developing leaf of the Semongok Emas variety (b), developing leaf of the Semongok Aman variety (c), mature leaf of the Kuching variety (d), mature leaf of the Semongok Emas variety (e), and mature leaf of the Semongok Aman variety (f). Scale bar = 4 cm.

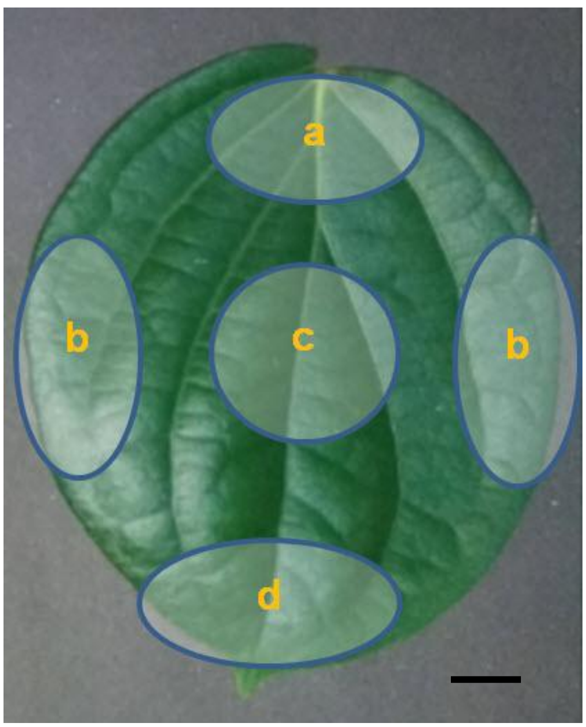

Figure 2. Four different parts of the leaf which were painted with nail polish on the (a) base, (b) side(s), (c) middle, and (d) tip. Scale bar = 2 cm.

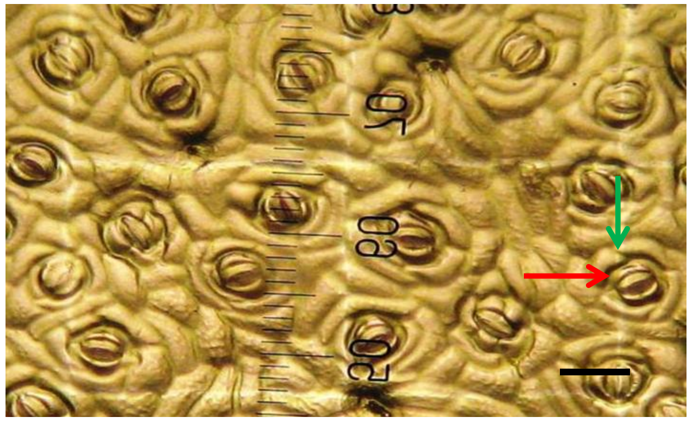

Figure 3. Stomata in the abaxial epidermis of *Piper nigrum* leaves observed under 40 x magnification with an image size of 0.515 mm x 0.386 mm. *Note.* Scale bar = 50 µm; Red arrow = a guard cell; Green arrow = a subsidiary cell.

The result for stomata distribution throughout the lower leaf epidermis revealed that except for mature leaf of the Kuching variety (KCH2), stomata were found abundantly at the base of the leaf in all varieties and developmental stages (Table 1). The reason for this was not entirely known and definitely not in agreement with a study done by Ravindram et al. (2005) which mentioned that stomata were evenly distributed throughout the epidermis without any definite pattern of orientation in *Piper* species. However, Boetsch et al. (1996) were of the opinion that the distribution of stomata on leaves is not random, but ordered. Bergmann and Sack (2007) and Boetsch et al. (1996) added that although the mechanism of stomata patterning was not known, environmental conditions such as the intensity and quality of radiation, humidity, temperature, carbon dioxide and ozone levels in the atmosphere, soil moisture and nutritional availability reportedly influence their frequency throughout the leaf epidermis. Since the growth of pepper lateral branches usually extend slightly upwards (Paulus and Sim, 2011), it may be the reason why stomata were mostly concentrated at the base of the leaves as it was the part most exposed to sunlight irradiance. This was in agreement with Martins et al. (2012) who mentioned that spatial distribution of stomata is also modulated by internal architecture and leaf position.

The stomatal densities in black pepper abaxial leaf surface based on its variety are shown in Table 2. *P. nigrum* of the variety Semongok Aman recorded the highest stomata count, followed by Semongok Emas and Kuching variety. Varieties which have wider and broader leaf base tend to have more stomata count. The result concurred with a leaf length to width ratio study done by Paulus and Sim (2011) indicating that the variety Semongok Aman possessed wider base area (1.52), followed by Semongok Emas (1.93) and Kuching (2.07).

Mean abaxial surface stomatal densities according to the developmental stages of black pepper leaf as summarised in Table 3 showed that developing leaves were found to have significantly higher stomatal densities than the matured leaves. This result suggested that epidermal cell development and stomatal development were regulated by different physiological mechanisms. Schletz (2008) discovered that developing leaves in plants tend to maximise growth potential by having higher gas exchange rates whereas mature leaves need to regulate water loss thus has a regulatory effect on its stomatal development.

Photosynthetic, transpiration and stomatal conductance of *P. nigrum* for var. Kuching, Semongok Aman and Semongok Emas showed significantly higher rates when readings were taken on the underside of the leaves (Table 4). These results indicated that stomata which were present under the leaves (abaxial) are responsible for carbon dioxide input and water output in black pepper, directly influencing carbon assimilation, plant water status and water use efficiency (Franks et al., 2017). Knowledge on the true position of the stomata can be very important for plant physiologists when doing gas exchange measurements. As it was discovered that the stomata in black pepper are only distributed underneath, some photosynthesis measuring machine will require the setting up of the protocol to read only at the underside of the leaves (abaxial) to get the optimum gas exchange values needed for more accurate measurements (LICOR, 2011). In terms of farm management, besides being a good entry point for foliar fertilisers for the plants to receive adequate nutrients (Schletz, 2008), the information also suggests that pepper is suitable to be planted in dry and hot areas of the tropic as the underside stomata are less exposed and protected from the sun thus reducing the rate of water vapor leaving (Ravindram, 2005).

Table 1. Stomatal densities according to specific area distribution in *Piper nigrum* leaves

Leaves	Adaxial surface stomatal density and distribution (count/mm^2)				Abaxial surface stomatal density and distribution (count/mm^2)			
	Tip	Side	Middle	Base	Tip	Side	Middle	Base
KCH1	-	-	-	-	25.33 ± 0.71^{a}	23.33 ± 0.97^{b}	19.50 ± 0.63^{c}	26.00 ± 0.86^{a}
SE1	-	-	-	-	27.00 ± 0.77^{a}	26.25 ± 1.04^{b}	25.75 ± 0.95^{c}	28.00 ± 0.79^{a}
SA1	-	-	-	-	29.33 ± 0.73^{d}	32.00 ± 0.90^{c}	33.67 ± 1.08^{b}	35.60 ± 1.14^{a}
KCH2	-	-	-	-	23.00 ± 0.94^{a}	21.00 ± 1.07^{c}	22.60 ± 0.93^{b}	22.60 ± 0.95^{b}
SE2	-	-	-	-	25.17 ± 0.99^{ac}	25.33 ± 0.82^{ac}	24.83 ± 0.91^{c}	26.17 ± 0.87^{a}
SA2	-	-	-	-	26.20 ± 0.86^{d}	28.40 ± 0.96^{c}	30.20 ± 1.14^{b}	32.83 ± 0.72^{a}

Means with same letter(s) superscript within rows are not statistically different using Tukey's at P> 0.05 probability level. The leaves were developing leaf of the Kuching variety (KCH1), developing leaf of the Semongok Emas variety (SE1), developing leaf of the Semongok Aman variety (SA1), mature leaf of the Kuching variety (KCH2), mature leaf of the Semongok Emas variety (SE2) and mature leaf of the Semongok Aman variety (SA2) (mean $\pm$ S.D., n = 12).

Table 2. Stomatal densities according to varieties in *Piper nigrum* leaves

Leaves	Adaxial surface stomatal density (count/mm^2)	Abaxial surface stomatal density (count/mm^2)
KCH	-	22.92 ± 0.97^{c}
SE	-	26.07 ± 1.04^{b}
SA	-	31.03 ± 0.90^{a}

Means with same letter(s) superscript within columns are not statistically different using Tukey's at P> 0.05 probability level. The leaves were leaf of the Kuching variety (KCH), leaf of the Semongok Emas variety (SE) and leaf of the Semongok Aman variety (SA) (mean $\pm$ S.D., n = 48).

Table 3. Stomatal densities according to the developmental stages in *Piper nigrum* leaves

Developmental stages	Adaxial surface stomatal density (count/mm^2)	Abaxial surface stomatal density (count/mm^2)
Developing leaf	-	27.65 ± 0.98^a
Mature leaf	-	25.69 ± 1.06^b

Means with same letter(s) superscript within columns are not statistically different using independent T-test (mean $\pm$ S.D., n = 144).

Table 4. Gas exchange rates of *Piper nigrum* leaves based on different stomata ratio measured using the LI-6400XT Portable Photosynthesis System

Treatment	Photosynthesis, *A* (µmol $CO_2/m^2/s^1$)	Stomatal conductance, *gs* (mol $H_2O/m^2/s^1$)	Transpiration, *E*, (mmol $H_2O/m^2/s^1$)
Stomatal Ratio = 1	10.45 ± 0.99^b	0.30 ± 1.13^b	1.89 ± 1.26^b
Stomatal Ratio = 0	12.06 ± 0.82^a	0.41 ± 1.14^a	2.74 ± 1.08^a

Means with same letter(s) superscript within columns are not statistically different using Tukey's at P> 0.05 probability level (mean $\pm$ S.D., n = 144). Stomata ratio = 1 refers to equal stomata on both side of leaf whereas Stomata ratio = 0 refers to stomata on only one side of leaf.

CONCLUSIONS

Stomata can be found abundantly on the underside or abaxial leaf surface in black pepper, and if they are open and high-pressured foliar fertiliser spray is directed inside the canopy to reach the underneath side of the leaves, this can be a good entry point for the nutrients. Furthermore, it was found that in all pepper varieties except for var. Kuching, stomata are densely distributed at the base of the lower leaf surface. Therefore, due to its wide leaf width, Semongok Aman variety exhibited the highest stomata count in its lower epidermis leaf surface. In all varieties, leaves during developing stage have more stomata count than the mature leaves. This is probably due to stomata regulatory control imposed by mature leaves to prevent excessive water loss. As for gas exchange study, by setting up the photosynthesis reading machine (e.g. LI6400XT Portable Photosynthesis System) to measure only one side of black pepper leaf will depict the true values of net photosynthetic, stomatal conductance and transpiration rates.

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