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Stomatal response of red chili (*Capsicum annuum* L.) due to water stress

Putra, G. M. D.[1,2], Sutiarso, L.[1*], Nugroho, A. P.[1], Ngadisih[1] and Chaer, M. S. I.[1]

[1]Department of Agricultural and Biosystems Engineering, Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia; [2]Department of Agricultural Engineering, Faculty of Food and Agroindustrial Technology, Universitas Mataram, Mataram, Indonesia.

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Abstract The results showed that the water requirement of red chili (*Capsicum annuum* L.) plants varied depending on the Kc value in the growth phase. Under conditions of 30% ETc (T1), the water demand ranges from 0.63-2.4 mm/day; at 100% ETc (T2), the value of water requirements ranges from 2.12-8.00 mm/day; and at 150% ETc (T3), the value of water requirements ranges from 3.17-12.00 mm/day. The provision of different water requirements produces different stomatal responses. The highest number of stomata in T3 treatment was 16.25, and the least in T1 treatment was 10. The highest stomatal density in T3, T2, and T1 treatments were 185.22 mm^{-2}, 136.78 mm^{-2}, and 118.54 mm^{-2}, respectively. The largest porous area in the T2 treatment was 145.14 μm^2, and the smallest in the T1 treatment was 113.17 μm^2. Meanwhile, the stomata area and stomatal index in the three treatments were almost uniform, ranging from 577.44 μm^2 to 629.36 μm^2 for the stomata area and 0.17-0.2 for index stomata. It showed that the number of stomata and stomatal density of T1 and T2 treatments were significantly differed from T3 treatment. Meanwhile, the stomata area, porous area, and stomata index were not significantly differed. Plant morphological data were obtained by T3 treatment that gave the best results; production weight of 17.8 g, leaf area of 23.4 cm^2, number of leaves 200, and plant height of 62 cm. The red chili plant in the greenhouse system was more adaptive to water drainage.

Keywords: Evapotranspiration, Stomata, Water stress

Introduction

The water scarcity problem is being experienced in various parts worldwide due to an imbalance in its supply and the increasing demand (Zubaidi *et al.*, 2020). Water supply in agriculture has become important because of the increasingly complicated competition. Furthermore, climate change sometimes causes floods and puddles that affect plant growth.

[*] **Corresponding Author:** Sutiarso, L.; **Email:** lilik-soetiarso@ugm.ac.id

Therefore, it is necessary to increase water efficiency to meet crop water demands.

Plants experience a water deficit when the supply is less than the amount needed. Water stress is characterized by decreased cell water content and loss of turgor, causing wilting, closure of stomata, and decreased cell enlargement (Ahanger *et al*., 2017). Moreover, excessive rainfall or flooding hampers harvests and causes severe damage to crops and economic losses (Rajanna *et al*., 2018).

Red chili (*Capsicum annuum* L.) is a horticultural plant useful while fresh or as processed products. Although red chili peppers are usually consumed fresh, cooked, or processed in powder form, many chili-based food products are becoming increasingly popular, such as hot sauces, pasta, purees, and pickles (Kelebek *et al*., 2020). Chili plants are usually planted in the transition between the dry and rainy seasons. The leaves turn yellow and wrinkle when planted in the dry season. Chili cultivation has several challenges, including fluctuating harvest prices, attacks by pathogens and pests, rain, and natural disasters. Some of these factors significantly affect national inflation in the agricultural sector (Pratama *et al*., 2021).

Many studies have discussed drought stress in plants based on morphological and physiological parameters. These parameters include plant growth, number of leaves, root length, leaf area, yield, water potential, relative water content, stomata reaction, photosynthesis, and osmotic adjustment (Bozkurt Çolak *et al*., 2021; V. Hernandez-Santana *et al*., 2017; Alghory and Yazar, 2019; Widiyono *et al*., 2020); Bhusal *et al*., 2019). However, few studies discussed the stomatal characteristics in treating excess water stress the value of evapotranspiration (ETc). Therefore, this study aimed to examine the characteristics of stomata in treating excess water stress and the effects on each period of plant growth.

Materials and methods

Study area

The study was conducted from June to September 2021 in the Special Region of Yogyakarta, Indonesia, geographically located in coordinates 7°50'23.1"S 110°22'47.5"E. The greenhouse had three acres and was laid in the area with an average temperature of 29.3°C, relative humidity of 74.5%, wind speed of 2.30 m/s, and light intensity of 11.54 MJ/m^2.

Experimental design and layout

Irrigation system

The irrigation system comprised a water source, a 125 W water pump, 220 Volts/50 Hz voltage, and maximum suction power of 7 m. The network is built with a drip irrigation system consisting of 12 holes measuring 7 mm, emitters measuring 7 mm, high-density polyethylene (HDPE) hoses, and manual valves. Moreover, the irrigation network has a control system comprising an Arduino UNO microcontroller for inputting the language program. The system also has a Real-Time Clock (RTC) for regulating irrigation timing through a solenoid valve that works on-off.

Experimental design

This experimental study was conducted in a greenhouse size of 3 x 3 meters, shown in Figure 1. A completely randomized design was employed using three treatments with five replications. The treatments consisted of 30% ETc (T1), 100% ETc (T2), and 150% ETc (T3), conducted on Ta-Nvi curly chili seeds produced by Scani Seed Indonesia. The chili plants were sown in bamboo racks until they were 30 days after planting (DAP) and then moved into polybags measuring 25 x 30 cm. The plants were flushed with 200 ml during the transfer and waited for one week to obtain uniformity.

Figure 1. Greenhouse with screen net walls

Plant water supply were calculated using the crop evapotranspiration Penman-Monteith equation recommended by FAO (Gavili *et al*., 2018):

$$ET_o = \frac{0.408\Delta(Rn - G) + \left(\frac{900}{T+273}\right)U_2(e_s - e_a)}{\Delta + \gamma(1 + 0.34U_2)} \quad (1)$$

Where; ETo=Reference evaporation (mm/day), Rn=net radiation at the crop surface (MJ/m^2/day), G=soil heat flux density (MJ/m^2/day), and T=mean daily air temperature (^oC). Also, U2=wind speed (m/s), ea=actual vapour pressure (kPa), es-ea=saturation vapour pressure deficit (kPa), Δ=slope of saturation vapour pressure curve at temperature T (kPa/^oC), and γ= psychrometric constant (kPa/^oC).

Crop evapotranspiration (ETc) was determined based on (Parmar and Tiwari, 2020):

$$ET_c = k_c x ET_o \quad (2)$$

Where; ETc = crop evapotranspiration; ETo = Reference evapotranspiration and Kc = crop coefficient dependent on season and plant growth period.

Study parameters

Data were collected on plant height, the number of leaves, leaf area, yields, stomatal number, size, density, and index. Plant height and number of leaves were measured using a ruler during growth once a week for 95 DAP from planting to the first harvest. All plant data were collected and averaged for each treatment. Yields were weighed using a digital scale with a capacity of 10 kg and averaged on all plants for each treatment.

The number of stomata that opened and closed on red chili leaves was counted in the observed field of view. Opened stomata and pores were measured on the stomatal and porous areas. Observation employed an Olympus CX23 microscope mounted with an Optilab 2.2 camera, with a magnification of 400 times. Optilab takes an image of the stomata and measures it using the Image Raster (IR) version 3 application calibrated for more accurate measurements. Stomata density was calculated using equation 3 proposed by (Suriani *et al*., 2019):

$$\text{Stomata density} = \frac{\text{Number of opening stomata}}{\text{Area of view}} \quad (3)$$

The stomata index shows the ratio between the number of stomata divided by the number of stomata and epidermis. The index was calculated by the following formula (Maylani *et al*., 2020):

$$\text{Stomata index} = \frac{\text{Number of opening stomata}}{\text{Number of epidermis cell + Number of opening stomata}} \quad (4)$$

Data analysis

Data analysis was performed using the Analysis of Variance (ANOVA) test with a 95% confidence level and continued with the Duncan Multiple Range Test (DMRT) test with a 95% confidence level for all three treatments. The test used Statistical Product and Service Solutions (SPSS) software (Version 20.0) to analyze the differences in each treatment.

Results

Calculation of plants' water supply

Red chili plants were placed in polybags at 35 DAP. During the growth period, chili plants experienced an initial phase of 25 days, a crop development phase of 35 days, a mid-season phase of 25 days, and a late season phase of 10 days. The total crop takes 95 days or approximately three months. The water supply varies depending on plant growth, while the demand increases from the initial to the mid-period. However, water requirements decrease in the late period during the plants' development and fertilization. The change in ETc for each plant period in various irrigation water treatments is shown in Table 1.

Tabel 1. Irrigation water requirement in each period

Date	Stage	Kc	Eto (mm/day)	T1 (mm/day)	T2 (mm/day)	T3 (mm/day)
June-July 2021	Init	0.40	5.29	0.63	2.12	3.17
July-August 2021	dev	0.85	6.50	1.66	5.52	8.28
August-September 2021	mid	1.20	6.67	2.40	8.00	12.00
End of September 2021	late	0.60	6.11	0.95	3.167	4.75

Differences in crop water requirements depend highly on the crop coefficient (Kc) value that describes the rate of water loss (transpiration). At the beginning of growth, the Kc value was 0.4, while the highest value was 1.2 in the mid-period. The value of ETc can describe the water requirement of plants.

The value of ETc in each treatment is different. For T1 treatment, total ETc required by red chili plants was 5.01 mm/day, for T2 treatment was 18.8 mm/day, and for T3 treatment was 28.2 mm/day. The irrigation water supply during the planting period is shown in Figure 2.

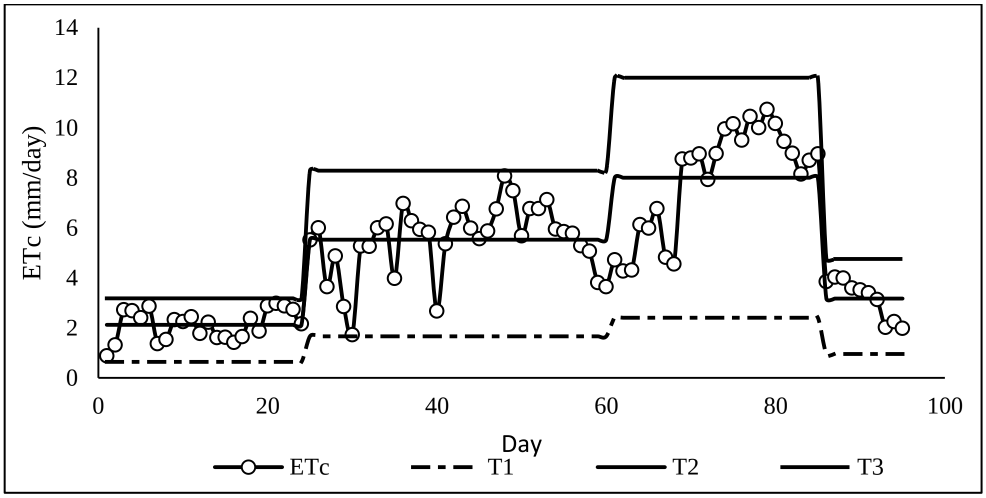

Figure 2. Irrigation water supply during the planting period

The analysis of the T1 treatment showed that the water requirement of the plants given was 30% lower than the T2 treatment for each growth phase. The value of plant water requirements at T1 range from 0.63 mm/day to 2.40 mm/day. T3 treatment showed a higher ETc value than T2 (Figure 2). The water requirement in this treatment was 150% higher than T2, with values ranging from 3.17 mm/day to 12 mm/day. The T2 treatment is a condition by giving water according to the plant's water needs (100%).

The irrigation water supply treatment depends on the ETc value in each period. Between the 1^{st} and 25^{th} day, the ETc T1 value was 0.63 mm/day, which was lower than the average daily ETc T2 value of 2.12 mm/day, and T3 had the highest ETc T2 value of 3.17 mm/day. Between the 26^{th} and 60^{th} day, the T1 ETc value was 1.66 mm/day, which was lower than the average daily ETc for T2 value of 5.52 mm/day; the T3 ETc value was higher than the T2 ETc value of 8.28 mm/day. Between the 61^{st} and 85^{th} days, the T1 ETc value was 2.40 mm/day, which was lower than the average ETc T2 value of 8.00 mm/day; the T3 ETc value was higher than the T2 ETc of 12.00 mm/day. Between the 86^{th} and 95^{th} days, the T1 ETc value was 0.95 mm/day, lower than the average daily T2 ETc value of 3.16 mm/day; the T3 ETc value was higher than the T2 ETc of 4.75 mm/day.

Red chili stomatal response

Red chili stomata (*Capsicum annuum* L.) have a wavy and irregular shape in the epidermis. The arrangement of epidermal cells is also tight, and there are no gaps between cells, as shown in Figure 3.

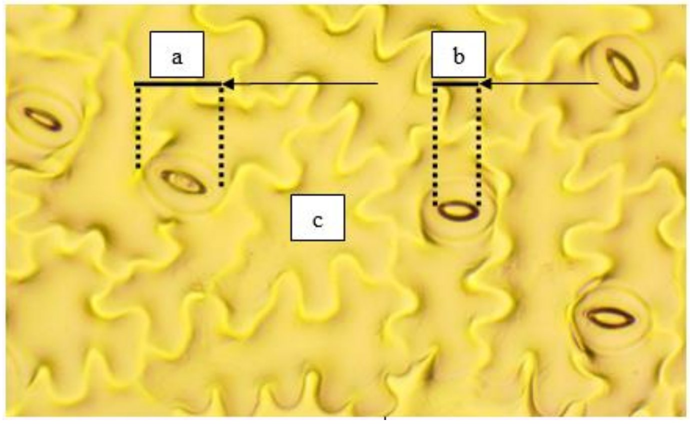

Figure 3. The shape of the epidermis and stomata of red chili leaves (a) stomata, (b) porous, (c) epidermis

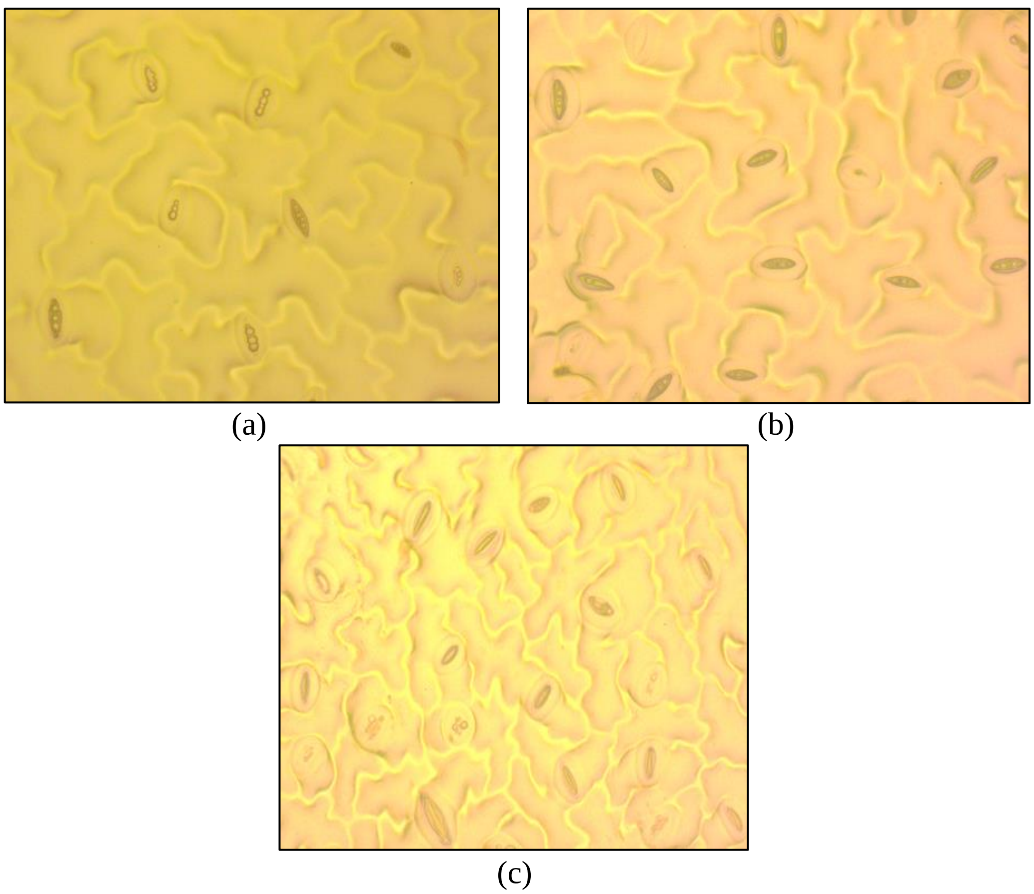

Figure 4. Characteristics response of stomata in treatment (a) T1, (b) T2, (c) T3

The epidermis tissue surrounds the stomata. These networks are separated from each other and determine the value of the stomatal index. Porous is located inside the stomata, so the size of the stomata is always more extensive than the porous. If it exposed to external stimuli such as sunlight or water availability, the number of stomata can change at any time, and the pore size can vary by opening and closing. Meanwhile, the size of the stomata tends to be similar. The characteristics of the stomatal response to treatment with various water levels are shown in Figure 4.

Result showed that the T1 treatment had the least stomata compared to the T2 and T3 treatments (Figure 4). Other parameters such as stomata size did not show much difference between treatments. The pore size in the T1 treatment was smaller than in T2 and T3, while the largest pore size was in the T2 treatment. The number of epidermis tended to be more in T3 treatment than in T1 and T2. The porous contain a bubble, which holds gas from the environment into the plant body or vice versa. The complete stomatal characteristic analysis data can be seen in Table 2.

Table 2. Characteristics of stomata in each treatment

Treatment	Number of stomata	Area of stomata (μm^2)	Porous area (μm^2)	Stomata density (mm^{-2})	Stomata index
T1	10.00±1.53a	577.44±19.39a	113.17±10.55a	118.54±17.41a	0.17±0.02a
T2	12.00±1.73a	629.36±41.44a	145.14±3.67a	136.78±19.74a	0.20±0.02a
T3	16.25±4.93b	578.80±17.69a	131.85±9.12a	185.22±56.22b	0.19±0.05a

Note: Mean±standard error. Different letters indicate a significant difference tested using one-way ANOVA and Duncan Multiple Range Test (DMRT)

The treatment of variations in water supply affected the stomatal response is shown in Table 2. The minimum number of stomata was 10 in T1 treatment, followed by 12 in T2 treatment, and the last was 6.25 in T3 treatment. The number of stomata linearly affects the density of stomata. T1 treatment revealed that the stomatal density of 118.54 mm^{-2} was the smallest compared to other treatments. Meanwhile, the T3 treatment had the highest stomatal density of the other treatments at 185.22 mm^{-2}. In the stomatal area parameter, the value is almost the same for each treatment, ranging from 577.4 μm^2 to 629.36 μm^2. The porous area gave different values for each treatment, where the T1 treatment had the least amount of 113.17 μm^2, and the most amount was found in the T2 treatment of 145.14 μm^2. Furthermore, the stomatal index in each treatment did not show a significant difference, with a value of 0.17 to 0.2.

An analysis was conducted using the F test with a 0.05 significance level and the difference in treatment with Duncan's test. The results showed a significant difference in the number of stomata in T3. Contrastingly, there was no significant difference between T1 and T2. Stomata density was significantly different in T3, with a value of 185.22. Moreover, the stomata area, index, and porous parameters were not significantly different in each treatment.

Red chili growth response

The morphological response (height, leaf number, leaf area, yields) of plant growth with three water supply treatments is shown in Table 3.

Table 3. Morphological responses of plant in each treatment

Treatment	Plant height (cm)	Number of leaves	Leaf area (cm^2)	Yields (gr)
T1	58.6±2.08a	118±15.28a	8.17±1.39a	2.8±1.00a
T2	64.4±1.53a	194±25.17a	13.63±1.51b	11.6±1.15b
T3	62±5.13a	200±15.28a	23.4±0.38c	17.8±5.51c

Note: Mean±standard error. Different letters indicate a significant difference tested using one way ANOVA fand Duncan Multiple Range Test (DMRT)

The plant height of red chili (*Capsicum annuum* L.) in T1 treatment was lower than in T2 and T3 treatments, at 58.6 cm, while the highest was found in T2 treatment, at 64.4 cm. For the number of leaves, it can be seen that the T1 treatment had the least number of leaves was 118; the number of leaves in the T2 and T3 treatments, respectively, were 194 and 200. As for the leaf area, the T1 treatment had the smallest leaf area of 8.17 cm^2, while the largest leaf area was in the T3 treatment, which was 23.4 cm^2. Provision of limited water, such as in T1 treatment, affects the production of red chili peppers with the lowest production value of 2.8 g, while T2 treatment with sufficient water produced 11.6 g. Finally, the T3 treatment showed the highest production of 17.8 g.

An analysis was conducted using the F test with a 0.05 significance level and the difference in treatment with Duncan's test. The results showed a non-significant difference for all treatment parameters of plant height and number of leaves. However, significant differences occurred in the leaf area and red chili production parameter.

Discussion

Chili plants have a small Kc value at the beginning of the growth period. This condition indicates the rate of water loss in the initial phase is less because the size of the plant and leaves are still small. In the development period, the value of Kc increases, meaning that in this process, the plant has grown large and has flowered and then fertilized so that the rate of water loss is the most. All of this describes the number of water plants needs to grow and develop. The late phase is the final phase, where the Kc value is lower than the development phase. In this phase, the plant has passed the productive period. ETc is directly proportional to Kc; the greater Kc, the greater the value of ETc, which is intended to maintain water balance in the plant body to support photosynthesis (Mushtaq *et al*., 2020).

Photosynthesis strongly influences plant growth (Harrison *et al*., 2020); it can run well if the stomata can carry out their functions properly. Stomata are located between epidermal guard cells and flanked by two neighboring cells. The plant tribe determines the number and arrangement of neighboring cells. They are scattered on the leaf surface in dicotyledonous plants, such as chili (Pautov *et al*., 2019). Stomata characteristics include their total number, density, index, area, and pores.

The number of stomata increases with the water supply, which effect is caused by environmental adaption, including water absorption by roots. Higher amounts of water absorbed by the roots increase the number of stomata in the plant. This increases the transpiration process by leaves to maintain water balance. Similarly, large stomata and wide leaves increase transpiration and photosynthesis rates (Saavedra *et al*., 2020). Environmental conditions with low humidity trigger water stress in plants, forcing them to use certain organs to grow normally (Lahive *et al*., 2019). The water stress condition in T1 reduces the number of stomata and transpiration.

The largest stomata area was 629.36 μm^2, found in T2, while T1 and T2 have 577.44 μm^2 and 578.8 μm^2, respectively. These results align with (Bhusal *et al*., 2020), which showed that the stomata area in excess water stress treatment is smaller than the regular water supply but does not differ significantly. In line with this, increasing the number of stomata increases the stomatal density. The stomatal and pore sizes are closely related. The pores in the stomata facilitate gaseous exchange and regulate the entry and exit of water (Urban *et al*., 2017). Larger stomata increase the porous size while opening, enhancing quicker gaseous exchange and higher transpiration. Stomata pores are responsible for removing water into the atmosphere through transpiration. Plants optimize CO_2 uptake for photosynthesis and minimize water loss by changing the stomata pore size (Bertolino *et al*., 2019).

Stomata density is closely related to plant resistance to drought stress. In this study, an increase in the number of stomata increases the stomatal density. This result supports Dittberner *et al*. (2018), which showed that stomata size and density have a strong negative correlation. The smallest stomata index of 118.54 in T1 treatment was the plant's adaptation to reduce transpiration. In contrast, the highest stomatal index of 185.22 in T3 increases transpiration. These results support other studies that decreased stomata index inhibits photosynthesis, transpiration, and CO_2 concentration between cells (Liu *et al*., 2020).

The high transpiration rate is because more water escapes increasing the absorption of nutrients from the soil. The absorbed nutrients are essential in increasing the photosynthesis rate, as well as plant growth and development. Conversely, a low transpiration rate reduces photosynthesis, carbohydrates, and crop yields (Omondi *et al*., 2019). Climate greatly affects the plants' physiological, where extreme environmental conditions increase various stresses (Raza *et al*., 2019). Under extreme water stress conditions, plants experience morphological, physiological, and development changes to survive and carry out their growth metabolism properly (Ahanger and Agarwal, 2017). The plant height of 58.6 in T1 was the lowest than T2 and T3. In line with this, previous studies found that drought stress significantly reduces plant height (Bhutta *et al*., 2019; Nasir and Toth, 2021; Bhutta *et al*., 2019; Jafari *et al*., 2019).

The water supply treatment in plants affects the number of leaves. The T1 results showed that the number of red chili leaves under water stress conditions was less than under normal conditions in T2 or decreased by 39.17%. The highest number of leaves in T3 was 200. These results support Pradhan *et al*. (2020), Descamps *et al*. (2018), Almaroai and Eissa (2020), and Anjum *et al*. (2017). The studies found that water stress significantly reduces the number of leaves than standard water supply conditions. Water stress inhibits cell expansion and division in leaf, stem, and root tissues. Morphologically, water stress reduces the area and number of leaves and turgor pressure (Hernandez-Santana *et al*., 2021). Another study found that a loss of turgor reduces the potassium content in the guard cells, causing stomata closure. This occurs when water loss by transpiration is not compensated for by water absorption (Time *et al*., 2018).

Plants that suffer from water stress have smaller leaf sizes. In Table 3, T1 and T2 had average leaf areas of 8.17 cm^2 and 13.63 cm^2, respectively. This result supports Bangar *et al*. (2019), which found a leaf area reduction in mung bean under drought conditions in the vegetative and reproductive phases compared to sufficient water conditions. Decreased leaf area increases plant resistance under water stress conditions due to decreased transpiration (Hopkins and Huner, 2018). Additionally, leaf area reduction is a mechanism to increase water use efficiency (Toscano *et al*., 2019).

The yield of red chili was the least in T1 compared to T2 and T3, which had a weight loss of 75.86% and 84.26%, respectively. This implies a relationship between stomata characteristics and production. In this case, more stomata and a wider porous area increase the plant yield. However, further studies should explore the negative impacts when plants continuously experience excess water stress.

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