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LEAF ANATOMICAL RESPONSES TO DROUGHT STRESS CONDITION IN HYBRID SUGARCANE LEAF (*Saccharum officinarum* 'KK3')

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ABSTRACT

Comparative leaf anatomy of sugarcane (*Saccharum officinarum* 'KK3') was investigated under early drought stress condition between 30-90 days of planting. Forty anatomical characteristics were studied using a light microscope with significance evaluated by numerical analysis. Results indicated that some anatomical features of sugarcane leaf responded to drought stress. Almost all the anatomical characteristics of unstressed treatments showed higher values than stressed treatments. A total of 21 out of the 40 characteristics showed a significant difference. Leaf thickness, stomatal size and interstomatal adaxial epidermal cell size of treated plants decreased while cuticle thickness of adaxial lamina and stomatal density significantly increased. This is the first report detailing leaf anatomy response to drought stress conditions in Thailand of sugarcane (*S. officinarum* 'KK3'). Results will provide useful basic knowledge for a better understanding of the adaptation mechanisms of tolerant sugarcane genotypes under early drought stress conditions.

Key words: sugarcane, leaf anatomy, adaptation, drought stress

INTRODUCTION

Sugarcane is an important economic crop grown in tropical and subtropical regions as raw material for the sugar industry, mulch or soil maintenance, energy industry, and surfactant industry (Wiedenfeld, 2000). Thailand is the fifth largest global sugarcane producer and the second largest sugar exporter (FAQ, 2016). In 2010, consumption of sugar in the country was estimated at 1.6-1.7 million tons, worth 17,000 to 19,000 million Thai Baht (Jangpromma *et al*., 2010). Most sugarcane production areas are planted under rainfed conditions. Crops grown during the late rainy season from October and November are known as over drought season plantations. However, rainfall distribution in Thailand varies by region and northeastern areas mainly comprise and sandy loams with lower rainfall and water absorption capacity. If the rains are late or rainfall is less than normal over the season, the sugarcane crop can be affected by stress conditions due to drought throughout the growing season resulting in reduced yield (Jaisil & Sanitchon, 2012; Jangpromma *et al*., 2012; Thongviang *et al*., 2014).

Previous reports recorded plants as showing responses to drought stress through both physiological and biochemical changes. Plants absorb water from the soil through their roots by osmosis to balance pressure changes. Free radicals induce damage to the plasma of cell membranes which allows leakage of electrolytes. This situation stimulates plant adaptation to promote survival (Boaretto *et al*., 2014). Plant cells respond by decreasing the water potential and carbon dioxide (CO_2) concentration in the mesophyll with reduced

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photosynthesis and growth rates (Shao *et al*., 2008). Anatomical responses include a reduction in leaf area and xylem size while pith increases and sclerenchymal cell walls become thicker (Bosabalidis & Kofdis, 2002). Leaf lamina and epidermis increase in thickness and bulliform cells expand, while stomatal size decreases, thereby increasing stomatal density (Nawazish *et al*., 2006). Plants in arid environments also showed xerophytic adaptation characteristics such as rolled leaf (Matsuda & Rayan, 1990), sunken stomata, thick epidermis, increase in bulliform cells and trichomes with multi-layers of mesophyll (Micco & Aronne, 2012). In particular, anatomical studies in sugarcane cv F127 and YL6 under drought stress conditions showed that bulliform cells of both cultivars decreased while adaxial and abaxial cuticles covering the epidermis thickened. However, the abaxial cuticle of cv YL6 decreased (Zhang *et al.,* 2015).

Interestingly, selections in sugarcane families appeared to be suitable for crop yield improvement when their physiological, morphological, bio-chemical and anatomical characteristics were considered. However, to our knowledge, no reports concerning anatomical characteristics relating to drought stress of *S. officinarum* 'KK3' exists. Cultivar KK3 is the most popular sugarcane variety grown in Thailand, representing 63% of the total crop and especially prevalent in the northeast region. The KK3 cultivar is a hybrid from clone 85-2-352×K 84-200 (Mother x Father). This cultivar has been identified as a drought tolerance genotype with high rationing ability (Office of the Cane and Sugar Board, 2015). Therefore, this research study investigated the responses of *S. officinarum* 'KK3' leaf anatomical characteristics under early drought stress conditions. Results will promote understanding to improve sugarcane genotypes in breeding programs for early drought resistance cultivars.

MATERIALS AND METHODS

Plant materials

Experiments were conducted on *S. officinarum* 'KK3' at the Field Crops Research Station, Faculty of Agriculture, Khon Kaen University, Thailand between March and December 2017. Two different water applications were used for moisture control as field capacity (FC) (non-water stress treatment) and water stress as water withholding during 30-90 days after planting. The soil moisture content measurements also confirmed adequate control of the irrigation applications (Figure 1). Amount of water supplied was calculated as crop water requirement based on Jangpromma *et al*. (2010) as follows:

$$ETcrop = ETo \times K_c$$

where ETcrop = crop water requirement (mm/day), ETo = evapotranspiration of a reference crop under specified conditions calculated by a pan evaporation method, and Kc = the crop water requirement coefficient for sugarcane. The amount of water supplied for well-watered treatment via this equation from 1-90 days after planting was 232.46 mm.

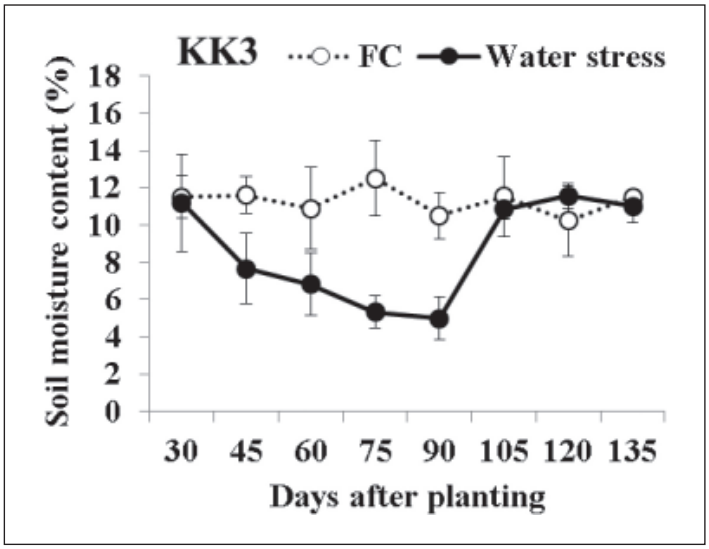

Fig. 1. Soil moisture contents percentage under two soil water managements of sugarcane (*S. officinarum* 'KK3') with drought and recovery periods. (Field capacity, FC; --○-- and drought at early growth stage, water stress; -●-).

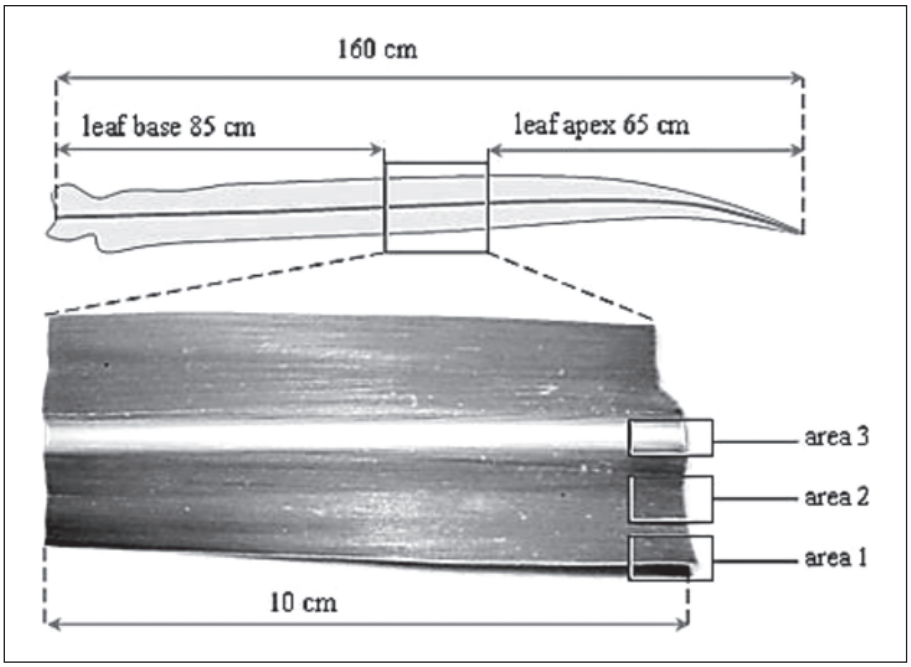

Fig. 2. Study areas of mature sugarcane leaf with selected cuts.

Mature leaves (third or fourth from shoot tip) were collected at 30 and 90 days after planting for both control and treatments. At least 160 cm long leaves were chosen and cut 2 cm above the ligule. A 10 cm of leaf blade was used for anatomical analysis (Figure 2).

Leaf anatomy analysis

Mature leaves of all treatments were dissected according to three areas of interest (Figure 2) into small pieces before soaking in 15% (v/v) sodium hypochlorite (Clorox) for 24 hours prior to peeling. Abaxial and adaxial epidermis were peeled and stained with 1% (w/v) Safranin O in 70% (v/v) ethyl alcohol) before preparation of a permanent slide using DePeX mounting medium. Stomatal size (including guard cell and subsidiary cell) and stomatal density were recorded under a light microscope. For leaf sectioning, three areas of the midrib, leaf margin and leaf blade of all treatments were transverse sectioned using freehand section technique and stained with 1% (w/v) Safranin O in 70% (v/v) ethyl alcohol before preparation of a permanent slide. Some anatomical characteristics were observed and scored using a light microscope (Olympus CH 30) and Zeiss 5402140000004 with MB2004 configuration-AxioVistion (MB2004 configuration-AV) program. Forty anatomical characteristics were recorded including lamina thickness, cell wall of epidermal cell and cuticle thickness of midrib (adaxial and abaxial), cell wall of epidermal cell and cuticle thickness of lamina (adaxial and abaxial), major vascular bundle in midrib (vertical length, horizontal length, diameter of the first metaxylem (vessel), diameter of the second metaxylem (vessel), protoxylem cell wall thickness (vessel), vertical length of phloem, horizontal length of phloem), length of bundle sheath extension of medium vascular bundle, major vascular bundle in lamina (vertical length, horizontal length, diameter of the first metaxylem (vessel), diameter of the second metaxylem (vessel), protoxylem cell wall thickness (vessel), vertical length of phloem, horizontal length of phloem), bulliform cell between large vascular bundle and medium vascular bundle (vertical length and horizontal length), stomatal density (adaxial leaf surface and abaxial leaf surface), stomatal size of adaxial leaf surface (width and length), stomatal size of abaxial leaf surface (width and length), interstomatal size of adaxial leaf surface (width and length), interstomatal size of abaxial leaf surface (width and length), short-cell size of adaxial leaf surface (width and length), short-cell size of abaxial leaf surface (width and length), long-cell size of adaxial leaf surface (width and length), and long-cell size of abaxial leaf surface (width and length) (Nawazish *et al*., 2006; Zhang *et al*., 2015).

Data analysis

Each treatment consisted of five replicates. Statistical analysis was conducted using the paired sample t-test to determine the significance of several variations in anatomical characteristics. The simple correlation was used to determine the relationship between anatomical characteristics.

RESULTS AND DISCUSSION

Peelings of control and treated leaves of *S. officinarum* 'KK3' showed epidermal characteristics of grasses with regular patterns of long cells and short cells. Types of tissues and their arrangements were similar for control and treated leaves. Leaf anatomy of sugarcane was described according to Metcalfe (1960) and Joarder *et al*. (2010). The abaxial epidermis exhibited short-cells over and between the veins; most of those over the veins were solitary and sometimes paired and arranged in short rows. Silica bodies were cross-shaped or intermediate between cross and dumb-bell shaped (Figure 3D); those between the veins being somewhat distorted in appearance. Stomata with triangular subsidiary cells were exhibited in the intercostal zone. Long-cells existed immediately beside and over the veins with thin to fairly thick sinuous walls; those in the stomatal zones were shorter and less frequent but with marked sinuations in the walls. Interstomatal cells or cells at the costal region had concave ends with very deep and narrow concavities.

Transverse sections of the leaf blades of control and treated *S. officinarum* 'KK3' exhibited the Kranz anatomy characteristics of C4 plants, with mesophyll cells radially arranged around the chlorenchymatous bundle sheath (Figure 4A-B). Forty anatomical characteristics were recorded. A total of 21 out of the 40 characteristics showed significant difference as lamina thickness, adaxial cell wall of epidermal cell and cuticle thickness of midrib, major vascular bundle in petiole (horizontal length, diameter of the first metaxylem (vessel), diameter of the second metaxylem (vessel), protoxylem cell wall thickness (vessel) and horizontal length of phloem), length of bundle sheath extension of medium vascular bundle, major vascular bundle in lamina (horizontal length, diameter of the first metaxylem, diameter of the second metaxylem, protoxylem cell wall thickness), vertical length of bulliform cell between large vascular bundle and medium vascular bundle, width of stomatal size of adaxial leaf surface, length of stomatal size of abaxial leaf surface, length of interstomatal size of adaxial leaf surface, length of interstomatal size of abaxial leaf surface, width and length of short-cell size of abaxial leaf surface, and width and length of long-cell size of abaxial leaf surface (Table 1).

Almost all the anatomical characteristics of unstressed treatments showed higher values than stressed treatments with the exception of five characteristics as adaxial cell wall of epidermal cell

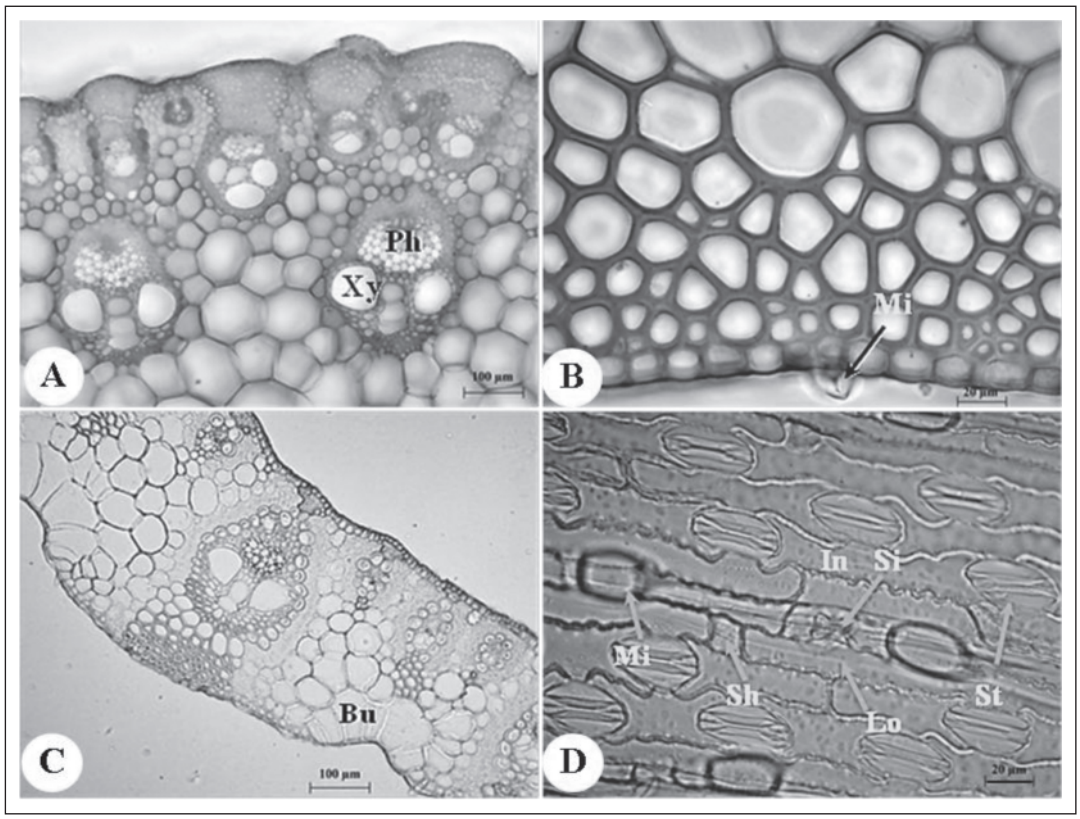

Fig. 3. Transverse section and peeling of sugarcane leaf. ***A***-major vascular bundle of midrib area; ***B***-fiber near adaxial surface; ***C***-major vascular bundle at lamina area; ***D***-epidermal cell and stomata at adaxial surface (*BC*-bulliform cell; *F*-fiber; *In*-interstomatal cell; *Lo*-long-cell; *Mi*-micro-hair; *Ph*-phloem; *Sh*-short-cell; *Si*-silica-body; *St*-stomata; *Xy*-xylem).

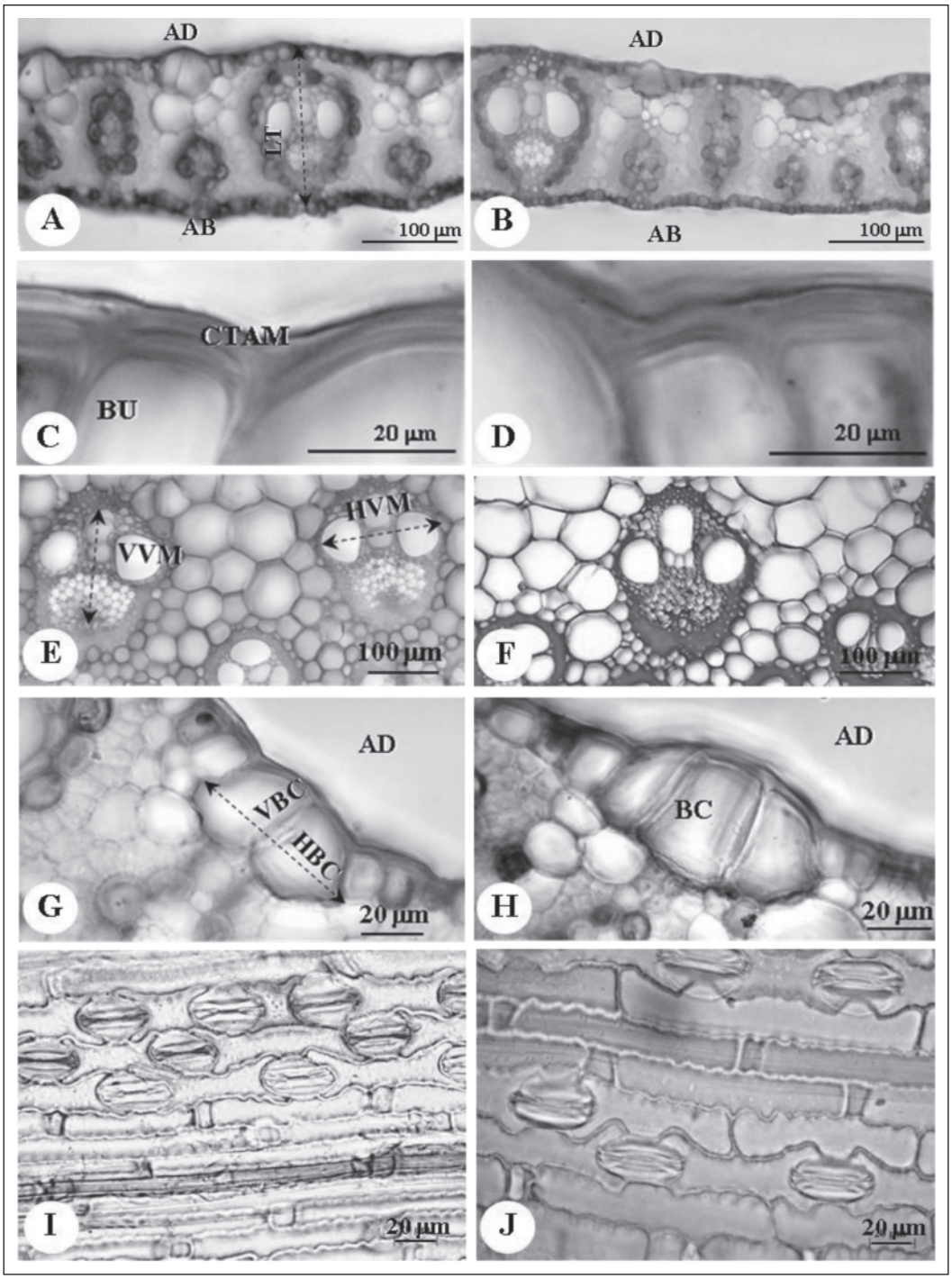

Fig. 4. Transverse section of sugarcane leaf showing the comparison of lamina thickness (LT) between field capacity (FC) and drought stress from 30-90 days after the first ratoon (D). ***A***-Field capacity; ***B***-drought stress; ***C***-cell wall of epidermal cell and cuticle thickness of adaxial midrib (CTAM) (FC); ***D***-cell wall of epidermal cell and cuticle thickness of adaxial midrib (D); ***E***-vertical length of large vascular bundle in midrib (VVM) and horizontal length of large vascular bundle in midrib (HVM) (FC); ***F***-vertical length of large vascular bundle in midrib and horizontal length of large vascular bundle in midrib (HVM)(D); ***G***-vertical length of bulliform cell (VBC) and horizontal length of bulliform cell (HBC) (FC); ***H***-vertical length of bulliform cell (VBC) and horizontal length of bulliform cell (HBC) (D); ***I***-stomata and epidermal cell at adaxial surface (FC); ***J***-stomata and epidermal cell at abaxial surface (D) (*AD*-adaxial; *AB*-abaxial; *CTAM*-cell wall of epidermal cell and cuticle thickness of adaxial midrib; *BC*-bulliform cell; *VBC*- vertical length of bulliform cell; *HBC*- horizontal length of bulliform cell).

Table 1. Comparison of some anatomical characteristics of sugarcane (*S. officinarum* 'KK3') leaf under well-watered (control) and water stress conditions

Characteristic	Well-watered (μm)(x ± SD)	Water stress (μm) (x ± SD)	Change (%)	Sig.
Lamina thickness	176.4 ± 2.9	143.0 ± 6.2	81.0	**
Cell wall and cuticle thick. of epi. cell Ad-midrib	4.5 ± 1.2	2.2 ± 0.6	48.8	*
Cell wall and cuticle thick. of epi. cell Ab-lamina	3.3 ± 0.8	4.9 ± 0.9	148.4	*
Horiz. length of major vas. bund. in midrib	157.9 ± 2.6	196.1 ± 3.1	124.1	**
Protoxy. cell wall thick (vessel). (midrib major vas. bund.)	4.1 ± 0.8	5.6 ± 0.5	136.5	**
Phloem horiz. length (midrib major vas. bund.)	116.8 ± 6.2	137.6 ± 1.4	117.8	**
Bundle sheath extension length	119.8 ± 0.9	145.9 ± 0.2	121.7	*
Lamina major vascular bundle horiz. length	96.6 ± 9.6	114.7 ± 5.1	118.7	*
BC vert. length	53.5 ± 3.6	41.7 ± 1.9	77.9	*
BC horiz. length	74.5 ± 5.2	51.7 ± 1.6	69.3	*
Interstomatal cell length (adaxial leaf surface)	22.5 ± 6.3	31.3 ± 3.6	139.1	*
Interstomatal cell length (abaxial leaf surface)	27.2 ± 5.3	45.9 ± 1.3	168.7	**
Short-cell width (abaxial leaf surface)	10.9 ± 1.3	15.1 ± 0.7	138.5	*
Short-cell length (abaxial leaf surface)	10.0 ± 0.9	7.8 ± 1.6	78	*
Long-cell width (abaxial leaf surface)	10.5 ± 0.8	15.2 ± 0.8	144.7	**
Long-cell length (abaxial leaf surface)	113.6 ± 1.5	91.4 ± 1.7	80.4	*
Stomatal density (Ad leaf surface) (No/mm^2)	216.2 ± 8.0	254.1 ± 1.6	117.5	–
Stomatal density (Ab leaf surface) (No/mm^2)	192.7 ± 9.2	183.9 ± 3.6	95.4	–

* Significant difference at $p < 0.05$.
** Significant difference at $p < 0.001$.
Ab-abaxial; Ad-adaxial; BC-bulliform cell; Epi.-epidermal cell; Horiz.-horizontal; Protoxy.-protoxylem; Thick.-thickness; Vas. bund.-vascular bundle; Vert.-vertical.

and cuticle thickness of midrib, vertical length of bulliform cell between large vascular bundle and medium vascular bundle, width of stomatal size of adaxial leaf surface, length of short-cell size of abaxial leaf surface, and length of long-cell size of abaxial leaf surface.

Some leaf anatomical characteristics of *S. officinarum* 'KK3' clearly showed responsiveness to drought tolerance as evidenced by decrease in leaf thickness, cell wall of epidermal cell and cuticle thickness of adaxial midrib, adaxial stomatal size, bulliform cell between large vascular bundle and medium vascular bundle (vertical length) and length of long-cell size of abaxial leaf surface. This result concurred with Zhang *et al*. (2015) who reported that cuticle thickness of the adaxial leaf epidermis was suitable to investigate sugarcane leaves of *S. officinarum* 'F127' and 'YL6' under drought stress. However, anatomical adaptation may differ between diverse species or cultivars (Graca *et al*., 2010).

Here, leaf thickness was significantly related to an increase of midrib vascular bundle, cuticle thickness and bundle sheath extension length of the major vascular bundle ($p < 0.05$). Moreover, leaf thickness positively related to decreasing adaxial epidermis stomatal size ($p < 0.05$). Decreasing leaf thickness was suitable for transpiration due to the reduction in leaf surface area. When the water content in plant cells is reduced, cells and cell walls are weakened as a result of decreasing cell volume and turgor pressure. If water deficiency occurs continually, cells become more packed with higher solute concentrations. This phenomenon triggers the sensitivity of plant growth under drought stress (Udomprasert, 2015). Shao *et al*. (2008) determined cell growth as the most sensitive process in the plant body.

Anatomical adaptation of plants with regard to water maintenance was clearly shown by the decrease in leaf area and a significant reduction in epidermal and mesophyll cell sizes ($p < 0.05$) while cell density increased, helping to reduce transpiration and respiration (Bosabalidis & Kofdis, 2002). Inhibition of cell expansion delayed leaf expansion and hampered the flow of hydrogen ions from inside to cell wall spaces under water deficiency conditions. This phenomenon resulted in non-difference in pH that was necessary for H-bonding to loosen small subunits of cellulose. Delaying cell and leaf expansion decreased the transpiration rate as a suitable mechanism to maintain water content in the plant cells for longer time periods. Reducing or limiting leaf area is an important adaptation under drought stress (Udomprasert, 2015). However, some reports showed that leaf thickness was positively related to plant growth and photosynthetic rate in drought stress condition. In most drought tolerant plants, leaf thickness increased when growing under drought

stress which caused an increase in mesophyll density (Kulya *et al*., 2014; Ngernmuen, 2013).

The stomatal density of *S. officinarum* 'KK3' was negatively related to stomatal width of the adaxial leaf and long-cell width of adaxial leaf surface but positively related to the short-cell size of the abaxial leaf surface. Stomata closed under drought stress condition to decrease the transpiration rate, resulting in lowering CO_2 fixation and photosynthetic rates (Taiz & Zeiger, 2002). However, the photosynthetic rate did not differ with decreasing leaf thickness, giving higher chloroplast concentration and increasing stomatal density while stomatal size decreased (Bosabalidis & Kofdis, 2002; Nawazish *et al*., 2006).

Stomatal responsiveness to drought stress differs in plant species. Drought stress cultivars showed higher photosynthetic rate and stomatal conductance than sensitive cultivars (Graca *et al*., 2010). Photosystem II (PS II) performance was stable until the last day of the experiment due to the protection of the protein involving PS II maintenance (Lu & Zhang, 1999). Therefore, increasing stomatal density and decreasing stomatal size generally assist in the photosynthetic process (Nawazish *et al*., 2006).

Major vascular bundles in midrib and lamina of *S. officinarum* 'KK3' leaf significantly increased after treatment ($p < 0.05$). Water and food transportation through tracheal elements were associated with photosynthesis. Water deficiency may interrupt transportation from source to sink because of the reduction in turgor pressure (Taiz & Zeiger, 2002). Vascular bundle sizes and bundle-sheath cells were positively related to photosynthesis and respiration rate (Wu *et al*., 2011) while increasing vascular bundle size caused an increase in water transportation performance (Bosabalidis & Kofdis, 2002).

CONCLUSIONS

Adaptation is important for plants to survive during drought stress conditions which impact on anatomical, morphological and physiological changes as well as at the genetic level. To reduce stress condition and still survive, increases in the cuticle, vascular bundle, stomatal density, leaf thickness and epidermal cells were shown to be necessary. Moreover, decrease in leaf area and stomatal size reduced photosynthetic rate, transpiration and other processes that protect plants concerning drought stress conditions. To our knowledge, this is the first report on leaf anatomy concerning drought tolerant sugarcane (*S. officinarum* 'KK3'). Results will provide useful basic knowledge for a better understanding of the adaptation mechanisms of tolerant sugarcane genotypes under early drought stress conditions. Further research is required to investigate variations of anatomical traits for other genotypes.

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