DOI: 10.1007/s11099-012-0055-1 PHOTOSYNTHETICA 50 (3): 458-466, 2012

Leaf cuticular waxes and physiological parameters in alfalfa leaves as influenced by drought

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Abstract

Drought significantly constrains higher yield of alfalfa (*Medicago sativa* L.) in arid and semiarid areas all over the world. This study evaluated the responses of leaf cuticular wax constituents to drought treatment and their relations to gas-exchange indexes across six alfalfa cultivars widely grown in China. Water deficit was imposed by withholding water for 12 d during branching stage. Cuticular waxes on alfalfa leaves were dominated by primary alcohols (41.7–54.2%), alkanes (13.2–26.9%) and terpenes (17.5–28.9%), with small amount of aldehydes (1.4–3.4%) and unknown constituents (4.5–18.4%). Compared to total wax contents, the wax constituents were more sensitive to drought treatment. Drought decreased the contents of primary alcohol and increased alkanes in all cultivars. Alkane homologs, C25, C27, and C29, were all negatively correlated with photosynthetic rate, transpiration rate, stomatal conductance, and leaf water potential. Under drought conditions, both stomatal and nonstomatal factors were involved in controlling water loss from alfalfa leaves. No direct relationship was observed between wax contents and drought resistance among alfalfa cultivars. An increase in alkane content might be more important in improving drought tolerance of alfalfa under water deficit, which might be used as an index for selecting and breeding drought resistant cultivars of alfalfa.

Additional key words: drought resistance; lucerne; photosynthesis; stomatal conductance.

Introduction

Alfalfa *(Medicago sativa)*, one of the most important perennial legume crops, is widely planted all over the world to improve the sustainability of agricultural systems and provides quality feed for livestock, particularly in arid and semiarid areas. However, periodic drought problems severely reduce alfalfa productivity all over the world (Aranjuelo *et al.* 2011).

Under drought stress, plants might rapidly downregulate photosynthesis *via* stomatal closure and thus minimize water loss and prevent cellular damage (Kottapalli *et al.* 2009). Bell *et al.* (2007) suggested that physiological and morphological adaptations to drought may be important in providing tolerance to water deficit. Among the morphological adaptations, leaf surface cuticular wax is thought to play a critical role in plant drought tolerance by reducing cuticular water loss and thus improve plant water-use efficiency (WUE) during water deficit (Samdur *et al.* 2003). Zhang *et al.* (2005) found that when WXP1, a gene able to activate wax production, was transferred into alfalfa, the transgenic alfalfa plants with increased cuticular waxes show reduced water loss and enhanced drought tolerance. Early studies also showed that increased amounts of cuticular wax were often associated with improved drought tolerance in oat (Bengston *et al.* 1978), rice (O'Toole *et al.* 1979), sorghum (Jordan *et al.* 1984), and crested wheat grass (Jefferson *et al.* 1989). However, Sanchez *et al.* (2001) found that only some pea cultivars showed a significant increase of epicuticular wax amounts under water deficit, indicating that cultivars within some species might have different mechanisms of wax deposition when plants are under water deficit. Similar results were also found in the studies of Kim *et al.* (2007) with

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Received 3 October 2011, *accepted* 15 April 2012.

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Abbreviations: BSTFA – N,O – bis (trimethylsilyl) trifluoro acetamide; DAT – days after drought treatment; FID – flame ionization detector; g_s – stomatal conductance; P_N – net photosynthetic rate; PPF – photosynthetic photon flux; RWC – relative water content; E – transpiration rate; WUE –water-use efficiency; Ψ_w – leaf water potential.

Acknowledgements: The authors are grateful to Lv Jun for his help and instruction using the Li-6400; Zheng Jun for his help in measuring wax constituents by GC/MS–GP2010. The study was supported by the National Natural Science Foundation of China for Young Scholars (30800802 and 31000122).

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sesame cultivars. All these studies show that the responses of leaf cuticular wax to water deficit depended upon genotypes of plant species.

It has been suggested that wax deposition on plant leaves can considerably affect the physiological activity of leaves including photosynthesis, transpiration, stomatal conductance and intercellular CO_2 (Mohammadian *et al.* 2007). Leaf cuticular waxes could help leaves retain water by minimising cuticular transpiration (Nautiyal *et al.* 2008). The wax coverage at the entrance of stomata in *Leucadendron lanigerum* increased resistance to gas diffusion and as a consequence decreased stomatal conductance, transpiration and photosysthesis (Mohammadian *et al.* 2007). The transgenic CER6:WXP1 alfalfa plants showed higher net photosynthetic rate, transpiration rate, and stomatal conductance under water stress and after rewatering (Jiang *et al.* 2009). Gonzalez and Ayerbe (2010) also concluded that a higher cuticular wax amount and a lower residual transpiration rate would confer a degree of drought tolerance to barley and help ensure better and more stable grain yields in areas where water is scarce at the end of the growing season. However, Sanchez *et al.* (2001) reported that the residual transpiration rate of pea cultivars was not correlated with its cuticular wax load, suggesting that the differences in residual transpiration among genotypes were due to other factors. No consistent relationship was found between leaf cuticular wax production and cuticular transpiration as estimated by water loss rate of excised leaves of alfalfa (Jefferson *et al.* 1989). The influence of leaf cuticular waxes on epidermal conductance also differed amongst species. Under water deficit, an increase in the quantity of waxes is negatively correlated with epidermal conductance in sorghum cultivars (Premachandra *et al.* 1992) and tobacco (Cameron *et al.* 2006).

Apart from the morphological adaptations to drought stress, plants will accumulate free proline in the leaf, acting as an osmoprotectant (Handa *et al.* 1983), thus helping maintain higher leaf relative water content at low leaf water potential (Blum 2005). Accumulation of proline in plant cells is believed to be related to mechanisms associated with tolerance to drought stress (Newton *et al.* 1986).

By comparing responses of biomass, survival, osmotic adjustment, and antioxidant enzyme activities to water deficit, alfalfa cultivars widely grown in China were classified into strong drought-resistant cultivars, such as Aohan and Daye, medium drought-resistant cultivar, such as Zhongmu, and low drought-resistant cultivars, such as Sanditi, Queen, and Defi (Han *et al.* 2006, Kang *et al.* 2004, Zhai *et al.* 2008). There is no information available on the influence of water deficit on alfalfa's wax profile, nor the relationship between leaf cuticular wax and physiological parameters related to drought stress. This study examines varietal differences in leaf cuticular wax amount and chemical composition on 6 alfalfa cultivars widely grown in China. The aim of the study is to analyse the responses of cuticular waxes to drought and to discuss the potential of using leaf cuticular waxes as an index for selecting and breeding drought-resistant alfalfa cultivar.

Materials and methods

Plants and drought treatment: Six cultivars of Medicago sativa were used, including Aohan, Daye, Zhongmu, Sanditi, Queen, and Defi. Ten seeds were sown in each pot (20 cm diameter and 20 cm depth) filled with a mixture of 2.0 kg of oven-dried soil and sand (2:1, v/v) and five living plants were kept when the first true leaf was emerged, replicated 8 times. The soil was a sandy loam purplish soil (typical Udorthent), containing 46.34 mg kg^{-1}(soil) of available N, 15.63 mg kg^{-1}(soil) of Olson P, 87.45 mg kg^{-1}(soil) of extractable K. Since no rhizobium was inoculated, ½ Hoagland solution was applied 10, 30, and 45 d after germination. All pots were watered (maintained at 75–85% field capacity by weighing the pots every day) and placed under a glass rain shelter. The temperature during the experimental period ranged from 10°C to 25°C. Sixty days after germination when plants were at branching stage (plant height averaged 40 cm), half of the pots were imposed with drought (no watering) and weighed every day to calculate the soil relative water content (RWC). The other pots were well watered every other day as control. Samples were used to measure leaf water potential, leaf free proline content, leaf gas-exchange indexes and cuticular waxes.

Leaf water potential (Ψ_w): The second fully expanded leaf from the shoot top of control and drought-stressed plants was cut and was measured for midday leaf water potential with a digital pressure chamber *1400* (*Skype Instruments*, UK) 6, 9, and 12 d after drought treatment when soil relative water contents were lower than 50% field capacity. Three leaves were measured in each pot.

Leaf free proline contents: Twelve days after drought treatment (DAT), about 0.3 g of leaf samples (third and fourth expanded leaf) were homogenized in 10 mL of 3% sulphosalicylic acid to measure the content of free proline spectrophotometrically following the method of Bates *et al.* (1973).

Leaf gas-exchange parameters: Twelve DAT, gas-exchange parameters were measured between 10:00 and 11:00 h in the third fully expanded leaf from shoot top using *LI–6400* Portable Photosynthesis System (*LI–COR Inc.*, Lincoln, Nebraska, USA). The leaves were measured under their respective CO_2 atmospheric concentrations during growth [400 μmol $mol^{-1}(CO_2)$] at 1,200 μmol m^{-2} s^{-1} PPF (photosynthetic photon flux) provided by a LED light. The temperature of the chamber was 18 ± 1°C.

Cuvette relative humidity condition for all treatments was 60%. Four leaves were measured in each pot. Gas-exchange parameters were calculated as described by von Caemmerer and Farquhar (1981).

Wax extraction and analysis: The third and fourth fully expanded leaves from the shoot top were sampled. A total of 10 leaves were sampled from five individual plants 12 DAT. Wax extraction was done according to the method of Kim *et al.* (2007). The leaves were dipped for 30 s into a 100-ml beaker with 20 ml hexane containing 0.2 mg hexadecane (GC grade) as internal standard. Extracts contained waxes from both abaxial and adaxial leaf surfaces. The extracts were evaporated to dryness under a nitrogen stream and the dried residues were derivated using 50 µL of BSTFA [N,O–bis(trimethylsilyl) trifluoroacetamide) for 20 min at 100°C, and surplus BSTFA was evaporated under nitrogen, the sample was redissolved in 1 mL of hexane for wax analysis using *GC–2010* (*Shimadzu Technologies Co*., Japan) equipped with a flame ionization detector (FID). The GC column was a 30 m × 0.25 mm × 0.2 µm *DM–5* capillary column and the carrier gas was nitrogen. The injector and FID detector temperatures were set at 300 and 320°C, respectively. The oven temperature of GC was programmed with an initial temperature of 80°C and increased at 15°C min^{-1} to 260°C, where the temperature remained unchanged for 10 min. The temperature was then increased at 5°C min^{-1} to 320°C, where the temperature was held for 15 min. Quantification was based on FID peak areas and internal standard. Compound identification was based on co-injection with commercial standards and analysis of a subsample in a *GC/MS–GP2010* (*Shimadzu Technologies Co*., Japan). The total amount of unknown constituents was calculated from the cumulative peak areas for all unidentified peaks. Total wax amount per unit leaf area was calculated as the total of all wax constituents including unknown peaks not identified by GC, and expressed as micrograms per total leaf area (µg cm^{-2}). After wax extraction, leaves were scanned and their surface areas were measured using *Adobe Photoshop CS3*. Leaf areas were calculated as the total of both abaxial and adaxial surfaces.

Statistical analysis: The data obtained were subjected to analysis of variance (*ANOVA*) using *GenStat Release 13.0* (*VSN International,* UK). The leaf physiological indexes and wax contents were tested by one-way *ANOVA* for significance between cultivars. The soil relative water content was analysed using two-way *ANOVA* for days after water termination and cultivar effects and their interactions. Mean values were separated by protected Fisher's least significant difference (LSD). The *Pearson* correlations between total wax contents and gas exchanges were determined using *SPSS 13.0 for Windows* (*SPSS*, Chicago, USA). The means and calculated standard error (SE) are reported.

Results

Soil relative water content (RWC) and leaf water potential (Ψ_w): RWC for all alfalfa cultivars decreased to lower than 50% six DAT, to less than 40% at day 9, and to less than 30% at day 12 (Fig. 1). RWC at day 9 and day 12 were the highest for Aohan and the lowest for Daye, but the difference was not significant between cultivars.

Six DAT, Ψ_w of all cultivars except Defi decreased significantly compared to the control ($p<0.05$) (Table 1). With increasing drought stress, Ψ_w showed decreasing trends, while significant change was only found for Aohan and Queen at 9 DAT, and for all cultivars except for Daye at 12 DAT.

12 DAT, Daye had the lowest Ψ_w (about –1.02 MPa) under control conditions.

Contents of leaf free proline: 12 DAT, drought-treated plants showed significantly higher contents of leaf free proline than those in control plants (Fig. 2). Under control conditions, contents of leaf free proline in Queen was the highest, followed by Defi, Zhongmu, Aohan, Daye, and Sanditi. Under drought treatment, content of leaf free proline in Sanditi was the highest, followed by Defi, Zhongmu, Queen, Aohan, and Daye.

Leaf gas exchange: 12 DAT, net photosynthetic rate (P_N) in all cultivars except Queen and Defi decreased significantly (Fig. 3). The transpiration rate (E) and stomatal conductance (g_s) of drought-treated Zhongmu and Sanditi significantly decreased, while there were no significant changes for the other cultivars. Sanditi showed relatively higher P_N, E, and g_s compared with other cultivars in well watered control plants. WUE, which was calculated as P_N/E, decreased for Aohan and Daye but increased for Sanditi 12 DAT. Aohan showed higher leaf WUE compared to other cultivars.

Leaf cuticular wax: Across all six alfalfa cultivars, the average of total leaf cuticular wax content of well-watered control plants was 12.92 µg cm^{-2} (Fig. 4). The waxes were mainly constituted with alcohols (41.7–54.2%), alkanes (13.2–26.9%), terpenes (17.5–28.9%), aldehydes (1.4–3.4%), and unknown constituents (4.5–18.4%). Alkane constituents on alfalfa leaves consisted of odd and even numbers of carbon atoms from C22 to C34, with odd-numbered compounds predominating, particular C27 and C29 (Fig. 5).

12 DAT, contents of total wax in leaves of Aohan significantly increased while those of Defi significantly

Table 1. Changes of leaf water potential (Ψ_w) in five cultivars of alfalfa after 6, 9, and 12 d of water termination. Values (mean ± SE, *n* = 4) followed by *the same letters* within each column (a–h) or row (A–C) are not significantly different at $p<0.05$ (LSD).

Species	Treatment	Ψ_w [MPa]		
		Day 6	Day 9	Day 12
Aohan	Control	-0.69 ± 0.03^{Af}	-0.76 ± 0.01^{Agh}	-0.77 ± 0.02^{Ade}
	Drought	-1.03 ± 0.03^{Cb}	-1.15 ± 0.02^{Bc}	-1.28 ± 0.02^{Aab}
Daye	Control	-1.05 ± 0.03^{Ab}	-1.05 ± 0.03^{Ad}	-1.02 ± 0.05^{Ac}
	Drought	-1.22 ± 0.05^{Aa}	-1.30 ± 0.01^{Aa}	-1.32 ± 0.05^{Aa}
Zhongmu	Control	-0.74 ± 0.04^{Aef}	-0.67 ± 0.04^{Ah}	-0.68 ± 0.01^{Af}
	Drought	-0.87 ± 0.03^{Bd}	-0.97 ± 0.03^{Bde}	-1.19 ± 0.05^{Ab}
Sanditi	Control	-0.84 ± 0.04^{Ad}	-0.89 ± 0.02^{Aef}	-0.76 ± 0.06^{Ade}
	Drought	-0.97 ± 0.02^{Bbc}	-0.99 ± 0.02^{Bd}	-1.37 ± 0.03^{Aa}
Queen	Control	-0.76 ± 0.03^{Aef}	-0.80 ± 0.02^{Afg}	-0.67 ± 0.02^{Bf}
	Drought	-0.99 ± 0.02^{Bd}	-1.29 ± 0.04^{Ab}	-1.39 ± 0.01^{Aa}
Defi	Control	-0.82 ± 0.03^{Ade}	-0.69 ± 0.06^{Ah}	-0.82 ± 0.03^{Ad}
	Drought	-0.89 ± 0.02^{Bcd}	-1.00 ± 0.03^{Bd}	-1.36 ± 0.05^{Aa}

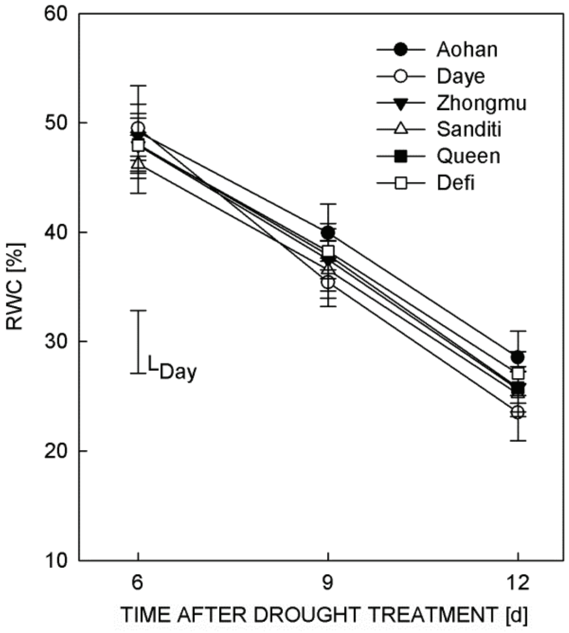

Fig. 1. Changes of soil relative water contents for 6, 9, and 12 days after water termination. *Vertical bars* show LSD (0.05) between days after water termination (L_{Day}). The values were the means of four replicates.

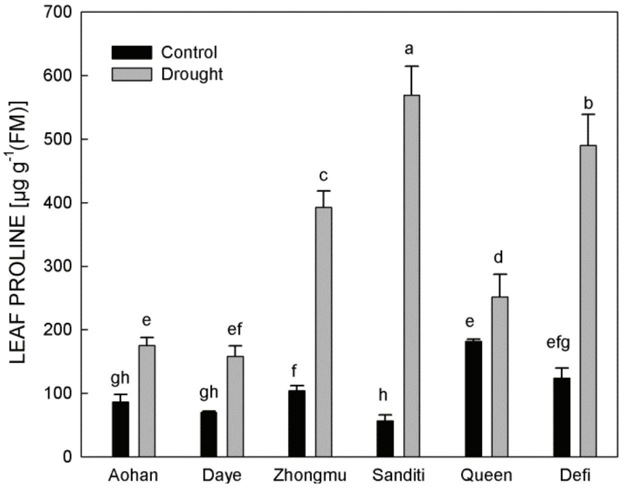

Fig. 2. Effects of drought stress on the contents of soluble proline in alfalfa leaves. Different *small letters* above the value bars (means ± SE, *n* = 4) are significantly different ($p<0.05$) according to least significant difference (LSD) test. FM – fresh mass.

decreased, with no significant change for other cultivars (Fig. 4). Compared to well watered plants, drought-treated plants had a 19–39% decrease in total alcohols, with a 36–78% increase in total alkanes. The responses of aldehydes, terpenes, and unknown constituents differed among alfalfa cultivars. In drought-stressed plants, contents of aldehydes increased by 1.5% and 14% for Zhongmu and Queen, decreased by 44% for Aohan, 63% for Daye, 40% for Sanditi, and 2% for Defi. Contents of terpenes in Aohan, Daye and Zhongmu increased by 49–72%, those in Sanditi, Queen, and Defi decreased by 35–49%.

Contents of unknown constituents increased in Aohan (61%), Zhongmu (79%), Sanditi (32%), and Queen (63%), and decreased in Daye (32%) and Defi (19%).

Under control conditions, the contents of total leaf cuticular waxes and alkanes in Aohan were significantly higher than those in Queen and Defi ($p<0.05$), followed by Daye, Zhongmu, and Sanditi ($p<0.05$) (Fig. 4).

Relationship between leaf cuticular waxes and Ψ_w and gas-exchange indexes: Ψ_w was negatively correlated with alcohols and positively correlated with alkanes and leaf free proline (Table 2). P_N was positively correlated with alcohols and aldehydes, negatively correlated with terpenes. E and g_s were only significantly correlated with alkanes. Among the alkane constituents, contents of C25, C27, and C29 were negatively correlated with P_N, E, and g_s.

Discussion

It has been believed that cuticular waxes on leaf surfaces played an important role in plant resistance to drought and an increased deposition of waxes on the surface of many plant species has been found after drought treatment (Jefferson *et al.* 1989, Kim *et al.* 2007). In this study, however, total wax content in one cultivar (Aohan) increased, in the other cultivar (Defi) decreased, and other four cultivars had no significant change. This was inconsistent with the studies of Zhang *et al.* (2005) and Jefferson *et al.* (1989), where drought treatment increased cuticular wax deposition on alfalfa leaves. The difference might be related to the genotypes used in the studies. Investigations of other researchers also found that leaf cuticular waxes deposition on plant leaves differed among cultivars and lines following drought treatment (Kim *et al.* 2007, Sanchez *et al.* 2001).

Compared to total wax content, the wax constituents in alfalfa leaves were more sensitive to drought treatment. Drought treatment caused a decrease of primary alcohol and increase of alkanes in all cultivars. Such responses indicated that wax constituents might be more important than total waxes in controlling water loss from leaves of alfalfa under drought conditions. On one hand, the changes in wax constituents might induce changes of wax crystalloid structures, thus alter E. Studies indicated that the chemical composition of cuticular waxes influenced wax crystalloid structure (Beattie and Marcell 2002, Koch *et al.* 2006). On the other hand, the increase of alkanes content in alfalfa leaves might increase the drought tolerance of plants. Kosma *et al.* (2009) reported that plant synthesized more alkanes to adapt to drought environment. Correlation analysis also showed that Ψ_w was significantly correlated with the contents of alcohols and alkanes in leaves of alfalfa.

However, the wax contents in alfalfa leaves and their responses to drought treatment were not consistent with drought resistance of the alfalfa cultivars. Queen and Defi, the low-drought-resistant cultivars, had higher contents of total leaf waxes than Daye and Zhongmu, the high and medium-drought-resistant cultivars. No significant relationship between total wax content and Ψ_w was observed. This indicated that wax deposition on alfalfa leaves was highly dependent upon plant genotype and the total wax contents showed no direct relationship with plant drought resistance. Under drought conditions, the content of terpenes in Aohan, Daye, and Zhongmu (locally bred cultivars) were significantly higher than those in Sanditi, Queen, and Defi. However, there was no significant correlation between the content of terpenes and Ψ_w. Whether such difference was related to their drought resistance is still uncertain.

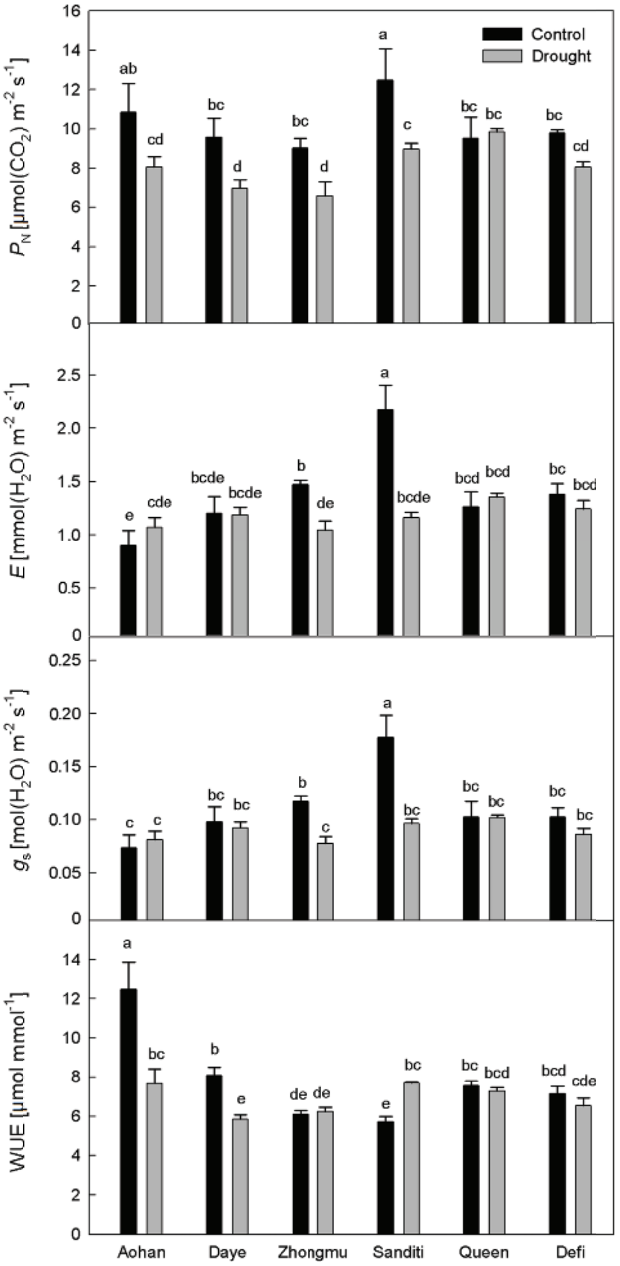

Fig. 3. Effects of water stress on leaf net photosynthesis rate (P_N), stomatal conductance (g_s), transpiration rate (E), and water-use efficiency (WUE) in six alfalfa cultivars. Different *small letters* above the value bars (means ± SE, $n = 4$) are significantly different ($p<0.05$) according to least significant difference (LSD) test.

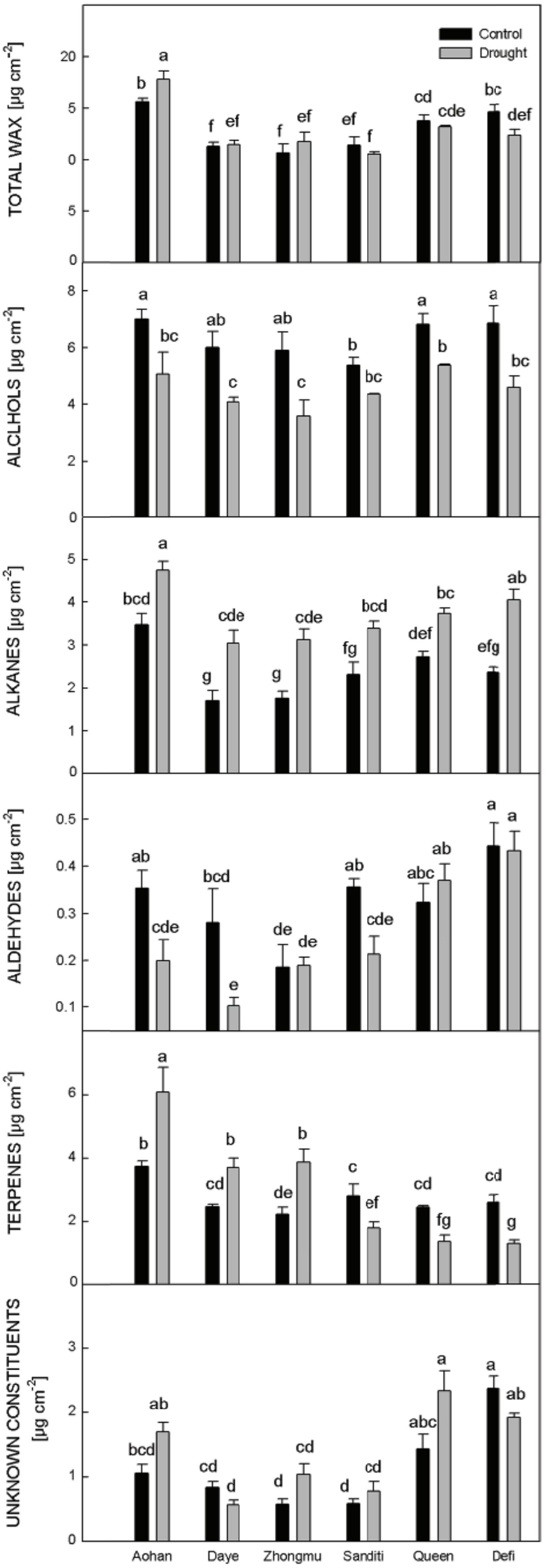

Fig. 4. The ranges of leaf culticular wax in six alfalfa cultivars across control and drought-treated plants. Total wax is the sum of primary alcohols, alkanes, aldehydes, terpenes, and unknown constituents. Different *small letters* above the value bars (means ± SE, $n = 4$) are significantly different ($p<0.05$) according to least significant difference (LSD) test.

Drought treatment caused a significant decrease of P_N for four alfalfa cultivars, the exceptions being Queen and Defi, while only Zhongmu and Sanditi showed significant decrease of g_s, indicating that there might be other factors limiting photosynthesis such as mesophyll factors as reported by Flexas and Medrano (2002). Dias and Brüggemann (2010) also concluded that the decrease of CO_2 assimilation under drought conditions was related to the decline of enzyme activity involved in RuBP regeneration. On the other hand, the responses of cuticular waxes to drought treatment might be also influencing gas-exchange indexes in alfalfa leaves. Mohammadian *et al.* (2007) reported that the wax coverage at the entrance of stomata in *Leucadendron lanigerum* increased resistance to gas diffusion and as a consequence decreased g_s,

Table 2. Correlation coefficients for leaf wax constituents and proline content with water potential and gas-exchange indexes across 6 alfalfa cultivars ($n = 48$). Ψ_w – leaf water potential, P_N – net photosynthetic rate, E – transpiration rate, g_s – stomatal conductance. ** $p<0.01$, * $p<0.05$, ns – not significant.

Wax constituents	Ψ_w	P_N	E	g_s
Total wax	-0.0709^{ns}	-0.0405^{ns}	-0.2974^{ns}	-0.3171^{ns}
Alcohols	-0.6562^{**}	0.3950^{**}	0.0614^{ns}	0.1029^{ns}
Aldehydes	-0.2424^{ns}	0.4430^{**}	0.2042^{ns}	0.1082^{ns}
Terpenes	-0.0030^{ns}	-0.3674^{*}	-0.2601^{ns}	-0.2474^{ns}
Unknown constituents	0.2110^{ns}	0.1991^{ns}	-0.0599^{ns}	-0.1371^{ns}
Alkanes	0.5769^{**}	-0.2979^{ns}	-0.4080^{*}	-0.4604^{**}
Alkane constituents				
C22	0.5265^{**}	-0.2423^{ns}	-0.3314^{*}	-0.4073^{*}
C23	0.5187^{**}	-0.2744^{ns}	-0.3619^{*}	-0.3886^{*}
C24	0.4311^{**}	-0.0342^{ns}	-0.1451^{ns}	-0.2028^{ns}
C25	0.4992^{**}	-0.4824^{**}	-0.4177^{*}	-0.4378^{**}
C26	0.5161^{**}	-0.0675^{ns}	-0.0996^{ns}	-0.1876^{ns}
C27	0.4750^{**}	-0.3874^{*}	-0.4869^{**}	-0.4943^{**}
C28	0.4134^{**}	-0.2146^{ns}	-0.3778^{ns}	-0.4333^{**}
C29	0.5451^{**}	-0.4194^{*}	-0.5131^{**}	-0.5324^{**}
C30	0.5133^{**}	-0.0967^{ns}	-0.2161^{ns}	-0.2949^{ns}
C31	0.4743^{**}	-0.1580^{ns}	-0.3603^{*}	-0.4253^{**}
C32	0.4819^{**}	0.0021^{ns}	-0.1290^{ns}	-0.2173^{ns}
C33	0.5238^{**}	-0.1514^{ns}	-0.2805^{ns}	-0.3536^{*}
C34	0.4503^{**}	-0.1145^{ns}	-0.2940^{ns}	-0.3586^{*}
Leaf proline	0.6252^{**}	-0.3186^{ns}	-0.2828^{ns}	-0.2964^{ns}

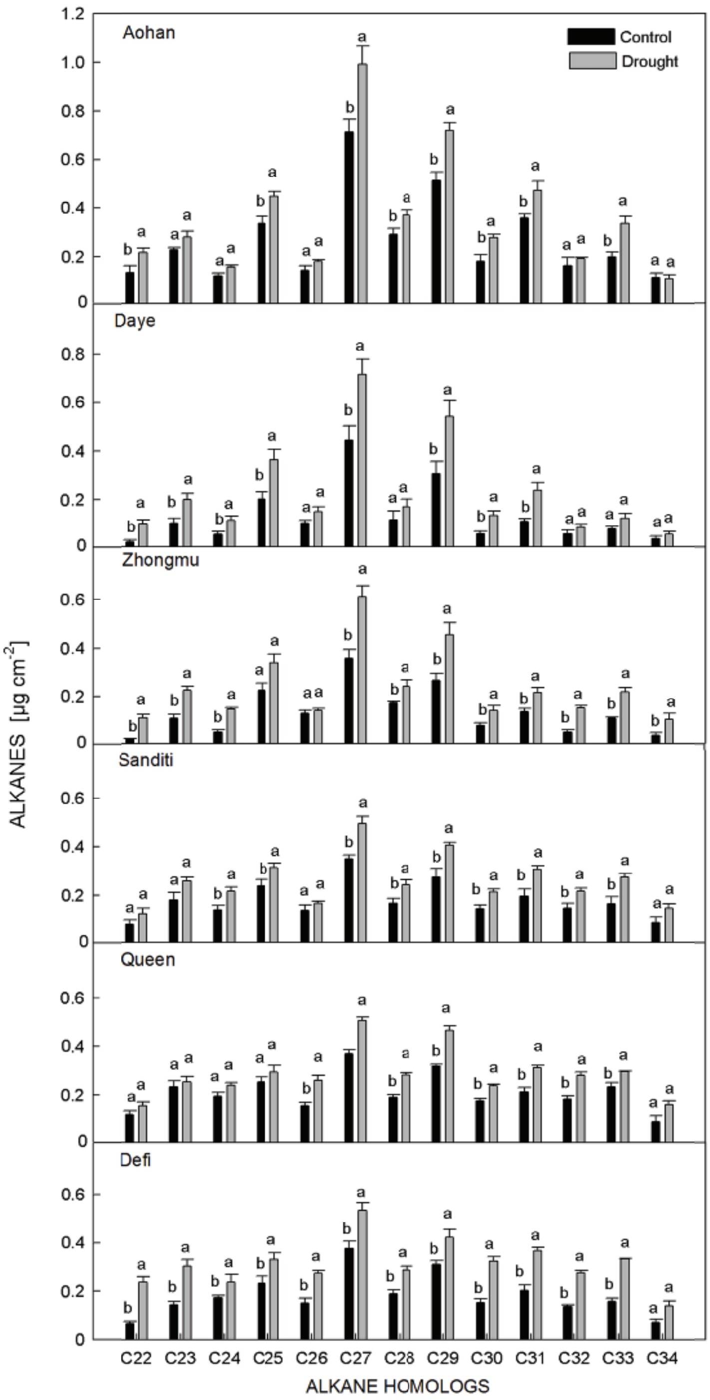

Fig. 5. Alkane constituents in leaves of different alfalfa cultivars as influenced by drought treatment. Different *small letters* above the value bars (means ± SE, $n = 4$) for each alkane homolog are significantly different ($p < 0.05$) according to least significant difference (LSD) test.

E, and P_N. Though total wax content was not significantly correlated with gas-exchange indexes, the contents of alcohols and aldehydes were positively correlated with P_N, while the content of alkanes was negatively correlated with E and g_s. This indicated that wax constituents rather than total wax content contributed to gas exchange. In alkane constituents, C25, C27, and C29 homologs were all negatively correlated with P_N, E, and g_s, suggesting that the increase of these homologs after drought treatment directly decreased water loss from the leaf surface. Studies have shown that cuticular waxes help leaves preserve water by decreasing cuticular transpiration (Jefferson *et al.* 1989, Kosma *et al.* 2009). Samdur *et al.* (2003) also reported that the cuticular wax on leaves of peanut reduced cuticular transpiration and improved WUE.

In this study, we also found that the leaf free proline, as an osmoprotectant, accumulated significantly after drought treatment, and its content was positively correlated with Ψ_w, indicating that osmotic adjustment also plays an important role on drought acclimation for alfalfas.

In summary, both stomatal (with cuticular waxes) and nonstomatal (with osmoprotectants) factors are involved in controlling water loss from alfalfa leaves under drought conditions. Though the drought resistance of the tested alfalfa cultivars was not consistent with their wax contents, an increase in alkane contents after drought treatment and their significant relationship with photosynthesis and transpiration rates imply that alkane constituents but not total wax contents might be used as an index for selecting and breeding drought-resistant cultivars of alfalfa.

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