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Effects of exogenous ascorbic acid on photosynthesis and xanthophyll cycle in alfalfa (*Medicago sativa* L.) under drought and heat stress

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Abstract: Alfalfa is an important leguminous plant, yield and quality depend on the growing environment, while effects of drought and heat stress on alfalfa leaves are unknown. This study was conducted to evaluate the effect of exogenous ascorbic acid (AsA) on photosynthesis, chlorophyll fluorescence, and xanthophyll cycle in alfalfa leaves subject to under drought and heat stress. The results suggest that drought and heat stress caused decreases in the net photosynthetic rate (P_n) in alfalfa leaves, but stomatal conductance (g_s), transpiration rate (T_r), and intercellular CO_2 concentration (c_i) were increased. The application of AsA could alleviate these changes to some extent. Besides, the decreased photosystem II (PSII) maximum photochemical efficiency (F_v/F_m) and violaxanthin (V) contents and significantly increased non-photochemical quenching (NPQ) levels. The increased NPQ corresponds to the de-epoxidation state (DPS) of xanthophyll pigments. In the AsA-pretreated alfalfa plants, the F_v/F_m and the NPQ were elevated, indicating that AsA could alleviate the adverse effects on photosynthesis induced by this stress. The violaxanthin de-epoxidase (VDE) enzyme activity was inhibited by drought and heat stress, and AsA significantly increased VDE enzymatic activity on the 2nd and 8th days. In summary, photoinhibition of PSII occurred in alfalfa leaves under drought and heat stress, resulting in decreased photosynthetic activity. Exogenous AsA can enhance the photosynthetic capacity of the plant, and enhance the drought and heat resistance of alfalfa.

Keywords: exogenous substances; abiotic stress; leaf gas exchange; climate change

Against the background of the rapid development of animal husbandry in China, forage quality and yield become more important. However, increasing drought and heat stress as a consequence of a changing climate can seriously impact the growth and development of forage (Li et al. 2000, Vinocur and Altman 2005). When solar energy exceeds plants' capacity, many physiological processes are impacted. However, under drought and heat stress, plants show various mechanisms to protect and enhance photosynthesis, such as heat dissipation, photorespiration, and xanthophyll cycling (Fahad et al. 2017, Liu et al. 2019).

Alfalfa (*Medicago sativa* L.) is commonly used as a basic feed in livestock production systems in more

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than 80 countries due to its rich nutritional value and dietary fibre (Darapuneni et al. 2020). Alfalfa is also a resilient species and can withstand prolonged drought by undergoing endo-dormancy, resuming growth when it receives sufficient water (Darapuneni et al. 2020). This is attributed to the deep root system of alfalfa, which not only improves soil fertility by symbiotic nitrogen fixation but also maintains soil and improves water use efficiency (Liu et al. 2023). Cv. Deqin alfalfa is a wild alfalfa distributed in Benzilan Town, Deqin County, Diqing Prefecture, Yunnan Province, China. Previous studies found that wild alfalfa showed strong characteristics of drought-heat-tolerant habitat conditions (Bi et al. 2007). After nearly 10 years of research and selection and cultivation, it was approved by the Grass Variety Certification Committee of the Ministry of Agriculture in 2010. Named *Medicago sativa* L. 'Deqin' (Announcement No. 1407 of the Ministry of Agriculture of the People's Republic of China). Although 'Deqin' alfalfa has a certain drought tolerance, when cultivated under drought and heat stress, it is negatively impacted. Thus, developing an understanding of the responses of alfalfa to drought and heat stress is crucial to improving yields.

When plants are subjected to environmental stress, photosynthesis is usually the first process to be affected (Sharma et al. 2015, Darapuneni et al. 2020). Drought and heat stress can impede photosynthesis by interfering with light energy capture, the PSII, the xanthophyll cycle and associated enzyme activity (Stasik and Jones 2007). On these grounds, photosynthesis has been used to detect and quantify damage in response to drought and heat stress in several crops, including wheat (Sharma et al. 2015), legumes (Teng et al. 2022), and maize (Li et al. 2018). Photosynthesis is generally the result of a combination of stomatal and non-stomatal regulation mechanisms. After stomata are closed, water loss is reduced, but stomatal closure also leads to a decline in intercellular CO_2 concentration (c_i) and photosynthetic rate (P_n). For example, stomata closure is the main physiological response of cotton to drought and heat stress, which is manifested by a decrease in the stomatal conductance (g_s), transpiration rate (T_r), and P_n (Hejnák et al. 2015). Xu et al.(2020) reported that the decrease in the P_n of alfalfa was influenced by stomatal and non-stomatal regulation mechanisms.

The PSII is the major contact point of plant photosynthesis and is linked to various photosynthesis-index physiological reactions, it can be damaged by strong light and heat (Dąbrowski et al. 2016). A decrease in the dark-adaptation maximum yield of PSII (F_v/F_m) indicates a photo-inhibition reaction to drought and heat conditions. PSII light-harvesting dissipated excess chlorophyll excitation energy in antennae through non-photochemical quenching (NPQ), one of the central photo-protective responses in most plants (Anaya et al. 2022).

Apart from the above mechanisms, plants can use the xanthophyll cycle to dissipate excess energy, thereby preventing an excessive reduction of the electron transfer chain as a result of thermal dissipation (Ruban et al. 2007). The de-epoxidation of V to antheraxanthin (A) under the action of excess energy is carried out by violaxanthin de-epoxidase (VDE). Subsequently, A is oxidised to zeaxanthin (Z), leading to the involvement of the PSII antenna in a mechanism that dissipates excess excitation energy (Qiu et al. 2003, Tang et al. 2012). The VDE enzyme activity is affected by light, which causes electron transport chains to form proton gradients on the thylakoid membrane, creating acidic lumens that allow enzymes to adhere to the membrane. However, the expression of the VDE gene is induced by light and drought (North et al. 2005). In tomatoes, overexpression of the VDE gene under chilling stress alleviates the photoinhibition of photosynthetic systems (Han et al. 2010).

Plants can also use a series of exogenous substances to resist abiotic stress, and the application of such substances is therefore an effective method to enhance plant resistance to drought and heat stress. Ascorbic acid (AsA) is an essential metabolite in higher plants. As an antioxidant, it frequently acts together with other components of the antioxidant system to protect plants from oxidative damage caused by photosynthesis and a range of abiotic stresses (Smirnoff et al. 1996). Chen et al. (2021) found that exogenous AsA could improve the AsA level in tomato seedlings under salt stress, thus reducing the photo-inhibition of PSII. In another study, exogenous AsA increased the contents of ascorbic acid, proline and pigments in tomato seedlings under heat stress, improved the activities of PSII and antioxidant enzymes, and reduced the oxidative damage to the photosynthetic system (Elkelish et al. 2020). Effects of AsA have also been observed under stress conditions induced by frost (Jalili et al. 2023), salt (Galicia-Campos et al. 2022), and heavy metals (Sharma et al. 2019), suggesting that AsA can directly affect the antioxidant capacity and photosynthesis in plants.

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Studies on the responses of legumes to drought and heat stress generally only focused either on drought or on heat stress. However, as both stresses have adverse impacts on the photosynthesis and the xanthophyll cycle of plants, their combined impacts on plants need to be considered. In this study, we hypothesised that ascorbate-treated alfalfa exposed to drought and heat stress induced systemic drought and heat tolerance, thereby reducing the damage caused by drought and high-temperature stress. Therefore, the effects of pre-treatment with ascorbic acid on photosynthesis, chlorophyll fluorescence, xanthophyll cycle and VDE enzyme activity of alfalfa leaves under stress were studied.

MATERIAL AND METHODS

Test materials and treatment. The 'Deqin' alfalfa seeds were obtained from the Laboratory of Grassland Science at Yunnan Agricultural University, Kunming, China. Alfalfa seeds were surface sterilised with 0.1% mercury hypochlorite for 30 min followed by repeated washing with distilled water. The washed seeds were germinated on glass Petri dishes with wet filter paper. On March 7, 2019, seedlings with consistent growth were selected and transplanted into plastic flowerpots containing 0.5 kg substrate (10 cm length × 10 cm width × 10 cm height). The culture medium consisted of fully mixed soil, laterite, and vermiculite (2:1:1), the soil nutritional characteristics were: 32.75% soil organic carbon, 259.00 mg/kg available nitrogen, 8.79 mg/kg available phosphorus, 55.80 mg/kg available potassium, pH of 7.56, and electrical conductivity of 1.88 dS/m. During cultivation, each pot was watered with Hoagland solution every 4 days until the plants grew to 10 cm high, and the seedlings with the similar growth were selected and transplanted into plastic flowerpots containing 1 kg substrate (20 × 20 cm). In the same medium, 20 alfalfa seedlings were planted in each pot. Then, the plants growing for 100 days were randomly selected and 18 pots (3 treatments × 6 replicates) of test materials with the same growth trend were treated according to the experimental design.

The drought and heat treatment started on June 19, 2019. There are three treatments: drought and heat stress (DH), drought and heat stress + AsA solution (DH + AsA) and control alfalfa (CK). For the DH + AsA treatment, the 5 mmol/L AsA solution was foliar sprayed with 100 mL per flowerpot, evenly spraying both sides of the alfalfa leaves, an equal amount of deionised water was sprayed on other materials, and then stress testing was performed. One day before the experiment started, we watered all the plants to about 50 mL and kept them to their maximum water content. Throughout the experiment, alfalfas in the DH and DH + AsA treatments will not be watered. In contrast, alfalfas in CK treatment are watered at 9:00 every day to maintain the relative soil water content in the pots at about 70–80% of the maximum field water capacity. The CK was cultivated in climate chamber (Ningbo Saifu Test Instrument Co., LTD, Ningbo, China) with a constant temperature of 22 °C, a photosynthetic photon flux density (PPFD) of 150 μmol/m^2/s, a day-and-night cycle of 14/10 h, and a humidity of 75%. The DH and DH + AsA were cultivated in a climate chamber at 32/28 °C (day/night), a PPFD of 150 μmol/m^2/s, a day-and-night cycle of 14/10 h, and a humidity of 30%. On the 1st, 2nd, 4th, and 8th days, and after rehydration on the 4th days, the relative index values of these three groups were determined.

Determination of parameters and methods

Leaf gas exchange. Mature alfalfa leaves (middle leaves of three split leaves) were labelled for each parameter determination. The gas exchange parameters of photosynthesis were measured using a LI-COR 6400 (LI-COR, Lincoln, USA) with an LED light source (light intensity of 400 μmol/m^2/s). Mature leaves from alfalfa with healthy and uniform growth were selected from the same leaf position and the parameters P_n, T_r, g_s, and c_i were determined. Flow rate, reference CO_2 concentration, and relative humidity were 200 and 400 μmol/m^2/s and 40–70%, respectively. Three plants were selected from each pot, the same leaves were taken, and each parameter was measured in triplicate for each plant.

Chlorophyll fluorescence. The same leaves selected for gas exchange parameters were used for measuring chlorophyll fluorescence parameters. The plants were placed in the dark for 30 min before determining the dark adaptation fluorescence parameters. The minimum fluorescence (F_0) was measured using a weak light of 0.5 μmol (photon)/m^2/s, and maximum fluorescence (F_m) was determined *via* a 0.8-s saturating flash of 8 000 μmol (photon)/m^2/s in dark-adapted leaves of alfalfa, followed by using the LI-COR 6400 with an integrated fluorescence chamber (LI-COR, Lincoln, USA). The steady state of fluorescence yield (F_s) was recorded, and a sec-

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ond saturating pulse at 8 000 μmol (photon)/m^2/s was applied to determine the maximal fluorescence yield of the light-adapted state (F_m'). The F_o' was the minimal fluorescence yield of the light-adapted state. Each parameter was determined in triplicate.

The maximum photochemical quantum yield of photosystem II was calculated as $(F_v/F_m) = (F_m - F_o)/F_m$ (Mattila and Tyystjärvi 2022). The photochemical fluorescence quenching efficiency (qP) was calculated from $qP = (F_m' - F_s)/(F_m' - F_o')$ (Van Kooten and Snel 1990). The coefficient of non-photochemical quenching of variable fluorescence (qN) was calculated from $qN = (F_m - F_m')/(F_m - F_o')$ (Shin et al. 2021). The electron transport rate was calculated as $ETR = (F_m' - F_s)/F_m' \times PAR \times 0.5 \times 0.84$ (Krall et al. 1992). The NPQ was calculated as $NPQ = F_m/F_m' - 1$, and $Y(NPQ) = F_s/F_m' - F_s/F_m$. The coefficient of photochemical quenching of variable fluorescence based on the lake model of PSII was calculated as $qL = qP \times F_o/F_s$. The above measurements were taken in an enclosed dark room. The xanthophyll components were extracted in a closed dark chamber. Briefly, 0.1 g of alfalfa leaves were frozen with liquid nitrogen and ground into a powder. Subsequently, 1 mL of 100% acetone was added, followed by homogenising for 2–3 min, placing in an ice bath for 15 min, and centrifugation (GL-20G-II Anting Scientific Instrument Factory, Shanghai, China) at 18 000 × *g* for 15 min.

Violaxanthin, antheraxanthin and zeaxanthin contents. The supernatant was tested for V, A, and Z contents *via* high-performance liquid chromatography (HPLC, Ultimate 3000 Thermo Fisher Scientific, Waltham, USA) as described in Xu et al. (2020). The conversion state of the xanthophyll cycle was calculated as follows: DPS = (A + Z)/(V + A + Z), according to Alonso et al. (2001).

VDE activity. The VDE enzyme activity was determined according to the instructions of the plant violet Xanthine decyclic oxidase (CsVDE) ELISA kit (Shanghai Enzyme Linked Biotechnology Co., LTD, Shanghai, China). Firstly, the standard product is diluted according to the corresponding concentration, and the blank hole (blank control hole does not add the sample and the enzyme-labelled reagent, the other steps are the same), the standard hole, and the sample hole to be measured are respectively arranged on the labelled plate. Add 50 μL standard product to the standard hole, add 40 μL sample diluent to the sample hole to be tested, and then add 10 μL sample to be tested (the final dilution of the sample is 5 times). Shake and mix, incubate at 37 °C for 30 min. Then 30 times the concentrated washing solution was diluted 30 times with distilled water for reserve use, each hole was filled with the incubating solution, and then discarded after 30 s, so repeated 5 times and then added 50 μL enzyme-labelled reagent was per hole, except for blank holes. Repeat the warming and washing steps. Colour developer A 50 μL was added to each hole first, and then colour developer B 50 μL was added to each hole, and the colour was gently mixed, and the colour was removed from light at 37 °C for 10 min, and the termination solution 50 μL was added to each hole to terminate the reaction (at this time, the blue turned to yellow). The wavelength of 450 nm was measured and the absorbance (OD value) of each hole was measured in sequence. The OD value of the sample is substituted into the equation, the sample concentration is calculated, and then multiplied by the dilution factor, that is, the actual concentration of the sample.

Total RNA extraction and determination of gene expression. The RNA was extracted from alfalfa leaves using a Spin Column Plant Total RNA Purification Kit (Huayueyang Biotechnology Co., Beijing, China), following the manufacturer's recommendations. The cDNA was synthesised by Prime Script reverse transcriptase according to standard protocols. Quantitative real-time PCR was implemented adopting the SYBR Premix Ex TaqII (2X), according to the manufacturer's instructions, using the following compounds: upstream primer F (10 μmol/L), 0.8 μL; downstream primer F (10 μmol/L), 0.8 μL; ROX reference dye II, 0.4 μL; template (cDNA), 2 μL; ddH_2O, 6 μL, with a total volume of 20 μL. The reaction conditions were as follows: one cycle at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, and 60 °C for 30 s. The primers for alfalfa VDE were AGTGCAGGATAGAGCTTGCG (upstream) and CGGGAGACTGCACACTCATT (downstream). For 18S rRNA, the primers were GAGAAACGGCTACCACATCCA (upstream) and CCCAACCCAAGGTCCAACTAC (upstream). Relative gene expression was calculated as described by Livak and Schmittgen (2001).

Statistical analysis. All data were analysed using the Statistical Package for the Social Sciences (SPSS Version 20.0, SPSS Inc., Chicago, USA) *via* one-way analysis of variance (ANOVA), and significant differences among means ($P < 0.05$) were determined by the Bonferroni test. Pearson's correlation between different concentrations of AsA and various levels of drought stress was calculated by SPSS software.

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RESULTS

Effects of exogenous ASA on photosynthetic gas-exchange parameters under drought and heat stress. Analysis of the photosynthetic gas-exchange parameters (Figure 1) showed significant changes under drought and heat stress. In contrast to the CK group, the P_n of the DH group was significantly reduced. Furthermore, the P_n of the DH + AsA group was significantly higher than that of the DH group, and spraying exogenous AsA significantly alleviated these reductions (Figure 1A). Both T_r and g_s showed similar trends in the DH group, after 2 days of stress treatment, the value was higher and decreased significantly from the 4th day, drought and heat stress caused these two parameters to be significantly higher than CK on the 2nd and 8th days. However, this increase was markedly alleviated by AsA treatment (Figure 1B, C). Interestingly, c_i showed an opposite trend to P_n, and c_i in the DH group was significantly higher than that in the CK group. However, this increase could also be mitigated by pretreatment with AsA (Figure 1D).

Effects of exogenous ASA on chlorophyll fluorescence under drought and heat stress. This study investigated the effects of drought and heat stress on various chlorophyll fluorescence parameters, including F_v/F_m, NPQ, ETR, qL, qN, and Y(NPQ), in alfalfa leaves, as well as the potential mitigating effect of exogenous AsA (Figure 2). The chlorophyll fluorescence parameters changed significantly under drought and heat stress. The F_v/F_m in the DH group started to decrease from 2nd days, and reached its minimum of 0.72 on 8th days, indicating an inhibition of PSII. However, this decline was markedly alleviated by AsA treatment for 2nd days. The NPQ value in the DH group was significantly higher than that in CK, reaching a peak of 1.33 on 8th days of stress treatment and returning to the original level after watering. Exogenous AsA significantly increased the NPQ value compared with

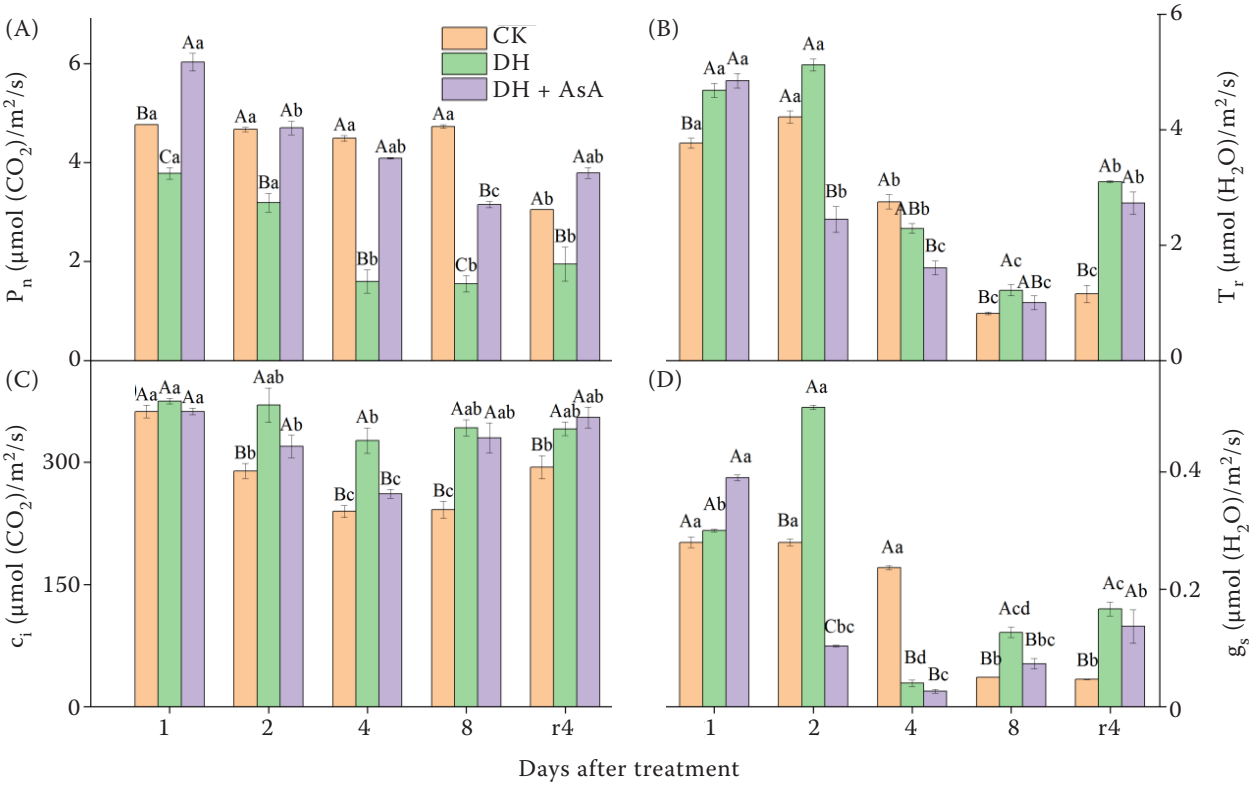

Figure 1. Effects of exogenous ascorbic acid (AsA) on photosynthetic gas-exchange parameters under drought and heat stress. (A) net photosynthetic rate (P_n); (B) transpiration rate (T_r); (C) intracellular CO_2 concentration (c_i), and (D) stomatal conductance (g_s). CK – normal growth environment; DH – drought and heat stress; DH + AsA – 5 mmol/ L AsA solution sprayed before subjection to drought and heat stress. All data are mean ± standard deviation. The data show averages from three independent repetitions and the error bars show standard error. Different lowercase letters indicate significant differences at different times in the same treatments ($P < 0.05$), and different uppercase letters indicate significant differences at the same time in different treatments ($P < 0.05$)

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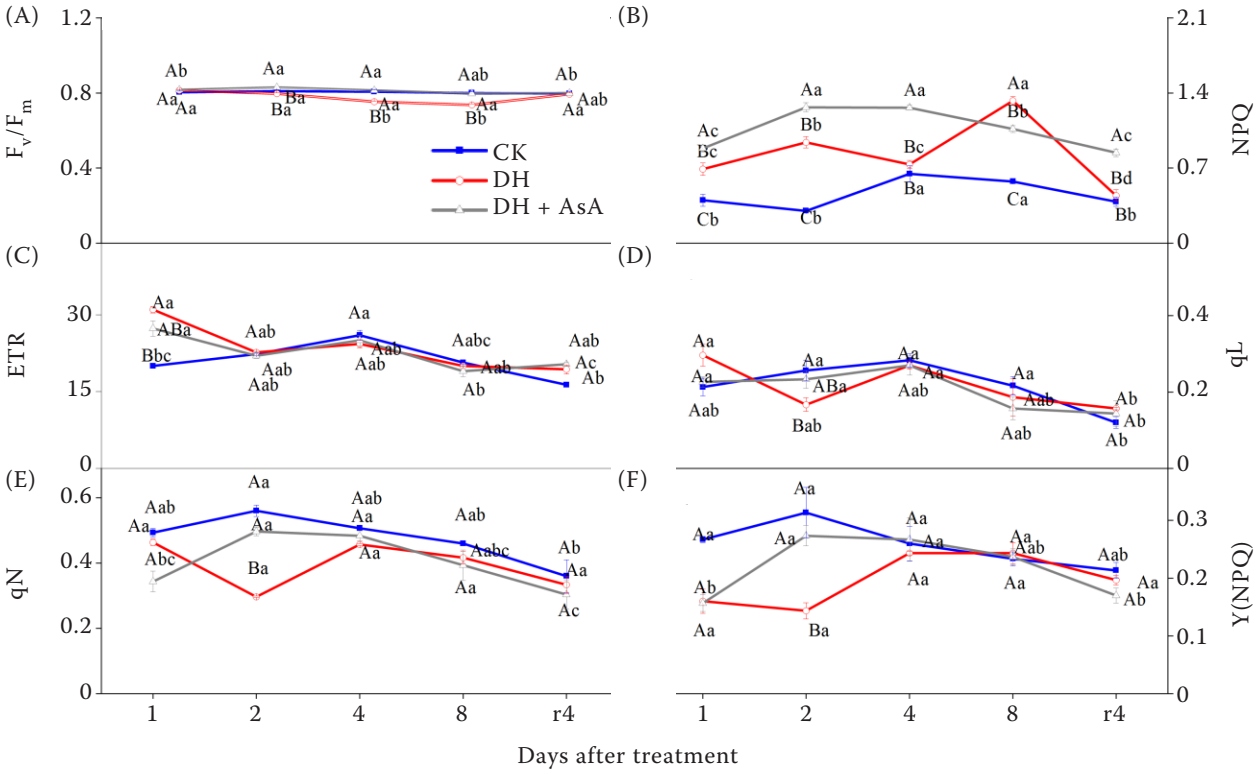

Figure 2. Effect of exogenous ascorbic acid (AsA) on chlorophyll fluorescence under drought and heat stress. (A) F_v/F_m, maximum photochemical quantum yield of PSII; (B) coefficient of photochemical quenching (qP); non-photochemical quenching (NPQ); (C) electron transport rate (ETR); (D) coefficient of photochemical quenching of variable fluorescence (qL) (E) coefficient of non-photochemical quenching of variable fluorescence (qN) and (F) Y(NPQ), yield for heat dissipationunder drought and heat stress. CK – normal growth environment; DH – drought and heat stress; DH + AsA – 5 mmol/L AsA solution was sprayed before drought and heat stress. The data show averages from three independent repetitions and the error bars show standard error. Different lowercase letters indicate significant differences at different times in the same treatments ($P < 0.05$), and different uppercase letters indicate significant differences at the same time in different treatments ($P < 0.05$)

the DH group. During the recovery phase, the F_v/F_m of the DH and DH + AsA groups recovered significantly, while the NPQ of the DH + AsA group sbarely recovered (Figure 2A, B). The variation trends of ERT in the DH and CK groups were similar but with a higher value in the DH group on 1st day (Figure 2C). The values of qL, Y(NPQ), and qN showed a similar trend. The qL, Y(NPQ), and qN values of alfalfa leaves of the DH group were significantly decreased on the 2nd day compared with those of the the CK group, which were decreased by 35.1, 54.3, and 11.3%, respectively. (Figure 2D, E, and F).

Effect of exogenous ASA on xanthophyll cycle under drought and heat stress. When alfalfa was subjected to drought and heat stress, significant changes in pigment distribution were observed (Figure 3). The V content decreased significantly in the DH group and the DH + AsA group as compared to the CK. However, this decrease was markedly alleviated by AsA treatment for 2nd days, the V content was increased significantly in the DH + AsA group, reaching 185 μg/g leaf FW (fresh weight) (Figure 3A). The content of A in DH group was significantly lower than that in CK group on 1st and 2nd days, and the reverse was observed on 4th and 8th days. Compared with DH, the A content in DH + AsA increased significantly by 32.0, 17.3, and 4.49% on the 1st, 2nd, and 8th days, respectively (Figure 3B). The Z content of the DH group was significantly lower than that of CK on 1st day and started to increase after 4 days of drought and heat stress treatment, reaching a value significantly higher than that of CK. Compared with DH, the Z content in alfalfa leaves in the DH + AsA group was significantly decreased after 2 days, namely by 11.7–6.8% (Figure 3C). Figure 3D shows the effects of drought and heat stress as well

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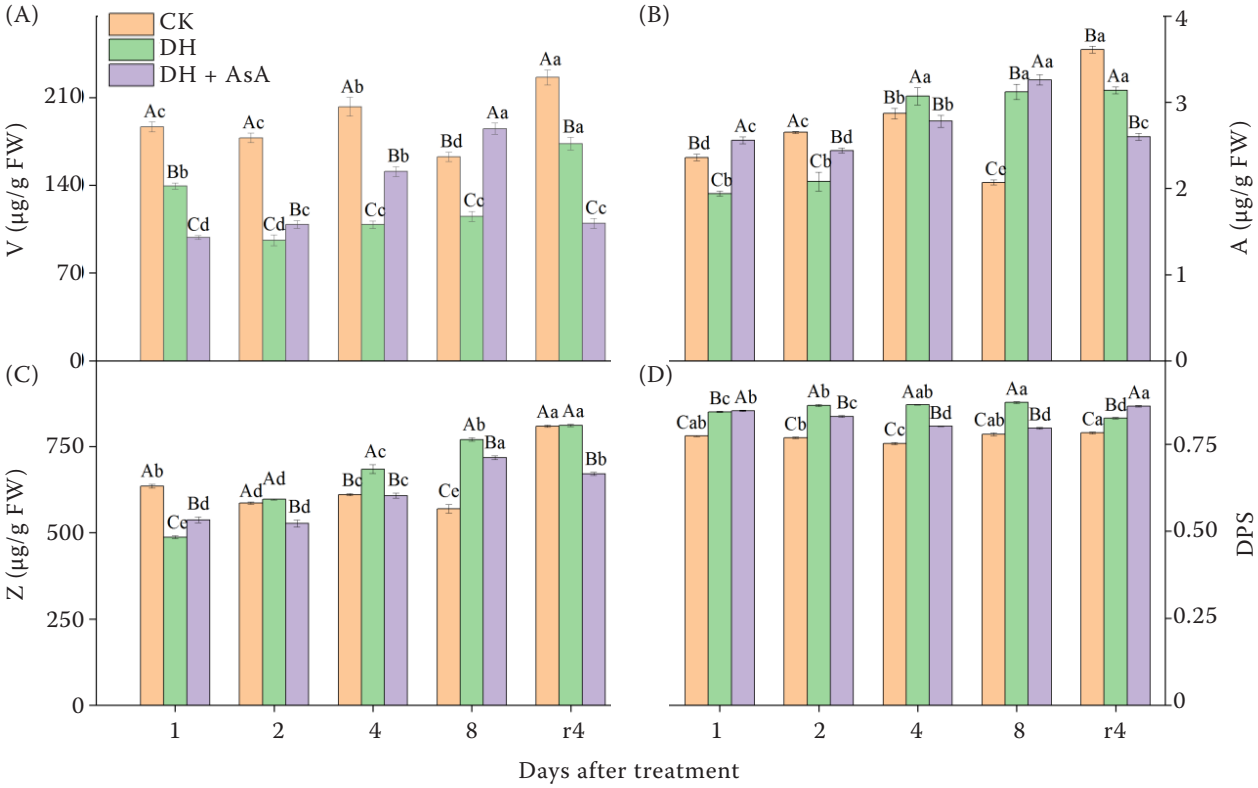

Figure 3. Effect of exogenous ascorbic acid (AsA) on xanthophyll cycle under drought and heat stress. (A) violaxanthin (V) content; (B) antheraxanthin (A) content; (C) zeaxanthin (Z) content, and (D) the conversion state of the xanthophyll cycle (DPS). CK – alfalfa growing under normal environment; DH – drought and heat stress; DH + AsA – drought and heat stress treatment after spraying exogenous AsA. The data show averages from three independent repetitions and the error bars show standard error. Different lowercase letters indicate significant differences at different times in the same treatments ($P < 0.05$), and different uppercase letters indicate significant differences at the same time in different treatments ($P < 0.05$); FW – fresh weight

as AsA spray on cyclic DPS in alfalfa leaves; the DPS values in the DH and DH + AsA groups were significantly higher than those in CK. The DPS in the DH group increased with the extension of stress time, reaching the highest value was 0.87 on the 8th day, and decreased by 5.25% after rehydration. The DPS in DH + AsA group was significantly lower than that in DH group on the 2nd, 4th and 8th days ($P < 0.05$).

Effect of exogenous ASA on VED enzyme activity and expression of VDE gene under drought and heat stress. The VDE activity and VDE transcription levels of alfalfa leaves under drought and heat stress changed significantly over time (Figure 4). The VDE activity of the DH group was significantly lower than that of CK, and there was a significant difference in VDE activity between leaves treated with DH + AsA and those treated with DH alone. The VDE activity of the DH + AsA group was significantly increased by 22.4% and 34.1% compared with that of the DH group on the 2nd and 8th days, respectively (Figure 4A). The expression of the VDE gene in alfalfa under severe drought and heat stress was significantly higher in the DH group compared with the CK group, and the expression of the VDE gene was increased by 114% and 97.2%, respectively (Figure 4B).

Correlation analysis. The P_n showed a significant positive correlation with F_v/F_m and negatively correlated with Z and A content. T_r showed a significant positive correlation with the two parameters: ETR and c_i, and negatively correlated with VDE transcript level and A content. The c_s showed a significant negative correlation with A content. c_i was significantly positively correlated with DPS, and negatively correlated with Y(NPQ). F_v/F_m and qL were significantly negatively correlated with Z content and VDE transcript level. NPQ was significantly positively correlated with DPS, and negatively

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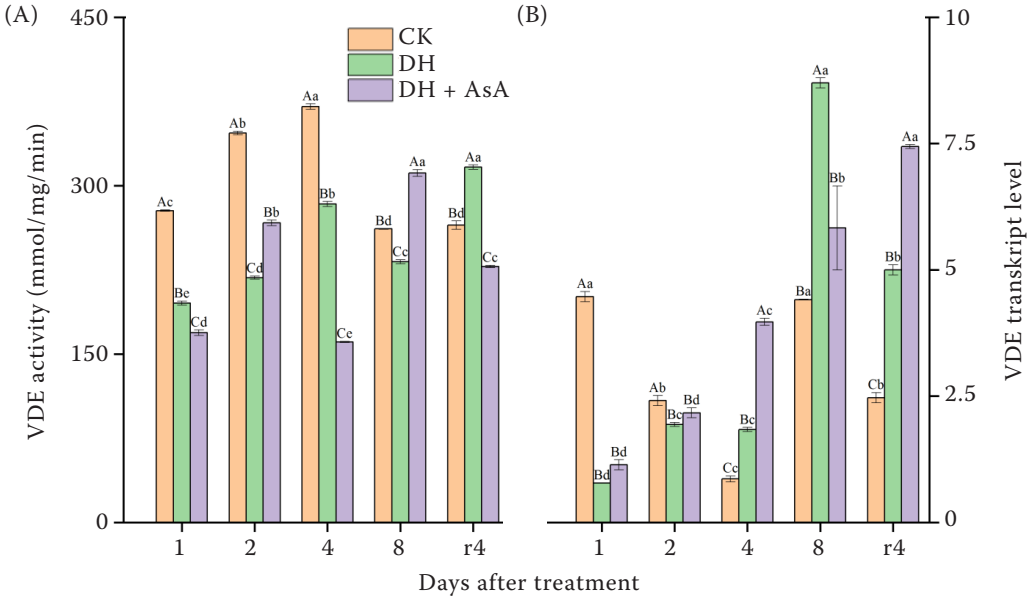

Figure 4. Effect of exogenous ascorbic acid (AsA) on violaxanthin de-epoxidase (VDE) enzyme activity under drought and heat stress. (A) VDE enzyme activity; (B) VDE transcript level. CK – alfalfa growing under normal environment; DH – drought and heat stress; DH + AsA – drought and heat stress treatment after spraying exogenous AsA. The data show averages from three independent repetitions and the error bars show standard error. Different lowercase letters indicate significant differences at different times in the same treatments ($P < 0.05$), and different uppercase letters indicate significant differences at the same time in different treatments ($P < 0.05$)

correlated with V content. ETR was significantly negatively correlated with A, Z content and VDE transcript levels. qN was significantly negatively correlated with DPS. Y(NPQ) and V content were significantly positively correlated with VDE activity, and negatively correlated with DPS. The A content showed an extremely significant positive correlation with Z content. The DPS showed a significant negative correlation with VDE activity.

DISCUSSION

Exogenous AsA increases drought and heat tolerance of the alfalfa by improving photosynthesis. Photosynthesis is a highly sensitive process in which the plant's metabolism needs to balance solar energy and energy expenditure. Environmental changes can easily disrupt this delicate balance, leading to significant changes in photosynthesis (Zhang et al. 2020). Drought and heat are two major abiotic stresses that often occur simultaneously, threatening crop production and biodiversity. The inhibition of plant photosynthesis under drought and heat stress is usually due to a combination of stomatal and non-stomatal factors (Xu et al. 2020). When the trends of P_n, g_s, and c_i are similar, this may have been caused by stomatal factors. However, when g_s decreases and c_i remains unchanged or increases, the decrease in P_n may have been caused by non-stomatal factors (Song et al. 2020). In the present study, after 1 day of drought and heat stress, the P_n decreased and the g_s and c_i increased (Figure 1), suggesting that non-stomatal factors were the main reason causing the decline in the photosynthetic function of alfalfa. Under various abiotic stresses, the photosynthetic efficiency of plants can be reduced by reducing the D1 protein turnover of the PSII reaction centre or by reducing the amount of photosynthetic pigments.

In a recent study, the decrease in maize photosynthesis under mild and moderate water stress was mainly caused by stomatal factors (Song et al. 2021). Tzortzakis et al. (2020) reported that stomatal restriction was not a major factor in changes in photosynthesis in Chardonnay grape plants. Rather, the different factors leading to the decline in photosynthesis may be related to the responses to stress of the different species as well as stress duration and intensity. Exogenous AsA supplied to drought- and heat-stressed plants promoted an elevation in P_n and decreased g_s and c_i. Photosynthesis reduction-

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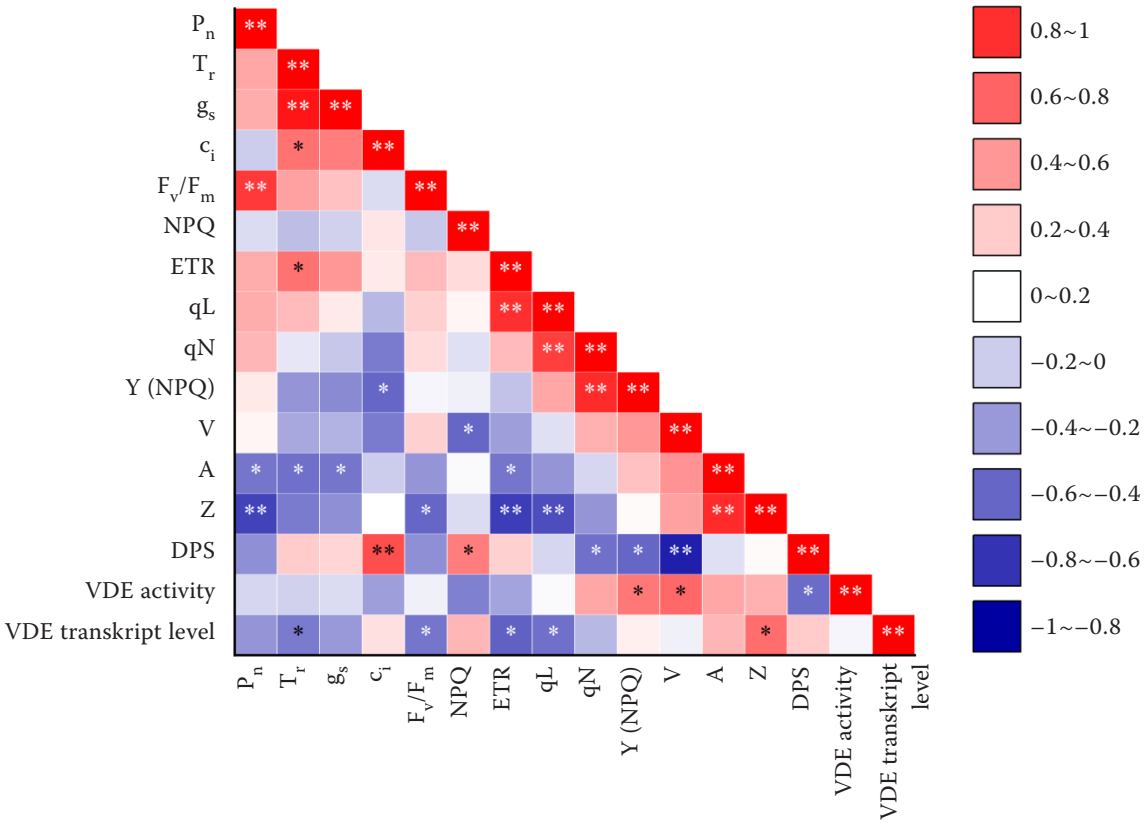

Figure 5. Correlation analysis. The corresponding value of the heat map is the Pearson correlation coefficient *r* (−1 to 1); $^{*}P < 0.05$; $^{**}P < 0.01$. P_n – net photosynthetic rate; Tr – transpiration rate; g_s – stomatal conductance; c_i – intercellular CO_2 concentration; F_v/F_m – photosystem II (PSII) maximum photochemical efficiency; NPQ – non-photochemical quenching; ETR – electron transport rate; qL – coefficient of photochemical quenching of variable fluorescence; qN – coefficient of non-photochemical quenching of variable fluorescence; Y(NPQ) – yield for heat dissipation; V – violaxanthin; A – antheraxanthin; Z – zeaxanthin; DPS – de-epoxidation state; VDE – violaxanthin de-epoxidase

induced drought and heat stress-induced reduction in g_s exposes chloroplasts to excessive excitation energy and induces oxidative stress by increasing the production of ROS such as H_2O_2. H_2O_2 is an important signalling molecule that promotes the closing of stomata. Plants with higher levels of AsA had a weaker response to the H_2O_2 signal and, because AsA can remove H_2O_2, exogenous AsA can reverse the stomatal closure induced by H_2O_2 (Elsiddig et al. 2022). This suggests that AsA maintains higher P_n levels by improving stomatal openness. In our study, the P_n of alfalfa under AsA treatment was higher than that in the middle and late stages of the drought and heat stress period, indicating that the addition of exogenous AsA improved the photosynthetic rate as well as drought and heat resistance.

Exogenous AsA can improve drought and heat resistance of alfalfa by improving photosystem II activity and xanthophyll cycle. The functions of the plant photosynthetic apparatus, such as the photosynthetic potential, the conversion of light energy into electron energy, and the level of plant photosynthetic activity, can be reflected in the chlorophyll fluorescence parameters (Liu et al. 2006). When plants suffer from severe drought stress, solar energy absorption and electron transfer processes are inhibited by a decrease in PSII activity (Murata et al. 2007, Liu et al. 2013). In the present study, although the ETR of alfalfa leaves was not significantly changed under drought and heat stress, a significant decrease was observed in F_v/F_m. The F_v/F_m ratio is an important index of the photochemical activity of PSII. It is generally employed to judge the extent of photo-inhibition of PSII (Bernardo et al. 2022). This suggests that the photochemical capacity of PSII in alfalfa leaves was inhibited under drought and heat

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stress. However, F_v/F_m returned to its original value at 4 days after resuming watering, suggesting that PSII inhibition was reversible at the given stress level. The treatment of alfalfa leaves with exogenous AsA could mitigate the decrease in the photochemical activity of PSII, indicating that exogenous AsA can reduce the photoinhibition of alfalfa leaves under drought and heat stress. The application of AsA to reduce stress damage and its effects on the photosynthetic apparatus has been reported previously (Alayafi 2020, Chen et al. 2023). Abiotic stress usually produces photoinhibition (Nawrocki et al. 2021), and the protective effect of NPQ on the photosynthetic apparatus is closely related to excess energy dissipation in non-radiative processes (Yang et al. 2011). This experiment shows that NPQ significantly increased under drought and heat stress, which is in agreement with the findings of Song et al (2013), who concluded that increasing NPQ is a mechanism for expending excess energy as heat (Tzortzakis et al. 2020). The application of AsA can significantly promote this increase, indicating a positive role of AsA in excess energy dissipation. These results are consistent with those reported for heat-stressed rice (Song et al. 2013). Exogenous AsA enhances the ability of alfalfas to converse and deplete light energy and enhance PSII activity during photosynthesis under drought and heat stress, suggesting that it plays a protective role in ameliorating photodamage.

In the xanthophyll cycle, especially in the DPS, Z is involved in the process of NPQ and dissipation of excess non-radiative energy. To verify this, we measured the variations in V, A, and Z contents under drought and heat stress (Figure 3). The xanthophyll cycle is an invertible process that promotes a balance between light absorption and heat dissipation to drive photosynthetic reactions. The DPS represents the de-epoxidation rate of the xanthophyll cycle in the positive reaction direction (Zhang et al. 2022). With the stimulation of the xanthophyll cycle pool by stress, the transformation of V towards A leads to the formation of Z. In our experiment, the V contents significantly decreased during drought and heat stress, while DPS significantly increased. According to a previous study, the NPQ relies on the xanthophyll cycle, and the increase of NPQ is attendant by the increase of xanthophyll save and preserve ability and DPS. This relationship was also confirmed through the correlation analysis (Figure 5). Moreover, the V content of the plants sprayed with exogenous AsA solution was significantly higher than that of the non-sprayed group after 2 days of drought and heat stress. The increase in the content of V may be considered an early light signal to protect the pigment-protein complex from stress-induced photodamage. We believe that the deep oxidation of alfalfa with exogenous AsA was delayed or accelerated, resulting in the accumulation of purple xanthine and lower DPS values. Xu et al. (2000) reported that AsA promoted the formation rate of Z, making the heat dispersive reliance on the xanthophyll cycle participate in photoprotection faster and more effectively. The same results were found for kidney beans under salt stress (Misra et al. 2006). Based on our findings, AsA regulation of the xanthophyll cycle largely controls the photoprotection of photosynthetic apparatus in alfalfa leaves.

Overexpression of the VDE gene can improve drought and heat resistance of alfalfa. The de-epoxidation of xanthophyll is catalysed by VDE, a core participant in the xanthophyll cycle. Our results show that the contents of DPS and Z in alfalfa leaves increased under drought and heat stress, which is in agreement with numerous earlier reports, while the activity of VDE, which catalyses the conversion to Z, decreased. The decreased VDE activity indicates the existence of a fast-acting control system that influences the integration or regression of VDE. Eskling et al. (1997) also recorded an inverse correlation between VDE and xanthophyll cycle pool pigments in spinach during the transition from low to high light; the authors observed that moving spinach from low-light to high-light environments resulted in a decrease in the amount of VDE and activity. In our study, the VED activity of alfalfa leaves treated with AsA was significantly higher than that of alfalfa leaves subjected to drought and heat stress only on the 2nd and 8th days. Enzyme activity can increase or decrease depending on a variety of factors, including transcriptional level, protein turnover, and cofactors. Although the VDE enzyme is an important enzyme that catalyzes xanthophyll cycling, it needs to bind to lipids, such as monogalactosyldiacylglycerol (MGDG) on the thylakoid membrane, to unfold its catalytic activity (Yamamoto and Higashi 1978).

Furthermore, the expression of *MsVDE* was also measured under drought and heat stress. In alfalfa leaves, *MsVDE* could be induced under drought and heat stress, and its expression levels increased, suggesting that it contributes to improving the ability of plants to cope with environmental stresses. In a previous study, overexpressing *MsVDE* could facili-

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tate the transformation of V from Z to A (Yang et al. 2015). The correlation analysis further showed that the transkript level of VDE gene was significantly positively correlated with Z content (Figure 5). The overexpression of *MsVDE* can moderate PSII photoinhibition by drought and heat stress by facilitating heat dissipation of the xanthophyll cycle. Han et al. (2010) showed that in tomatoes, the photoinhibition of PSII and PSI was alleviated by the overexpression of the VDE gene during high-light and chilling stress. So far, it has been established that overexpression of *MsVDE* genes can ameliorate the photoinhibition of PSII and PSI in plants under various stress conditions. In our study, the expression levels of the above genes increased slightly in AsA-treated plants under drought and heat stress, with a significant increase on the 4th day. This indicates that AsA played an important role in eliminating the harmful effects of early drought and heat stress on plants.

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