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Research Article

Effect of Acetic Acid Pretreatment on Drought Stressed Alfalfa Plants

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ABSTRACT

Drought stress is one of the major factors that reduce plant growth and productivity. This study was conducted to investigate the effect of exogenous acetic acid pretreatment on drought stress tolerance response in plants. Fourteen-day-old alfalfa plants were pretreated with 15 mM acetic acid, and then subsequently subjected to drought stress for 6 days. The fresh weight and relative water content in the leaves of acetic acid pretreated alfalfa plants were increased compared to the control group. The chlorophyll and carotenoid contents were slightly decreased in the acetic acid treatment. The H_2O_2 and proline contents were also significantly decreased in the acetic acid treatment. These results suggest that the scavenging mechanism of reactive oxygen species in alfalfa activated by acetic acid pretreatment is involved in conferring tolerance to drought stress.

(**Key words**: Acetic acid, Alfalfa, Drought stress)

Ⅰ. INTRODUCTION

Drought is the most important abiotic stress for crops and has a significant impact on plant growth and production (Fathi and Tari, 2016). Drought which are occurring more frequently due to climate change are expected to become more severe (Mukherjee et al., 2018). Grasslands provide numerous benefits to agriculture such as maintaining ecosystem stability, improving nutrient cycling and sequestering carbon (Gaujour et al., 2010). However climate change such as drought has a negative impact on the yield and quality of grasslands, resulting in the reduction of grasslands stability (Tubiello et al., 2007). Therefore development of forage crop plants with drought stress tolerance is necessary to ensure a stable supply of feed in the future.

Plants can alleviate water shortage to some extent through osmotic regulation which helps to maintain proper functions of plants under drought stress (Barker and Caradus, 2001). So far several approaches such as treatment with osmoprotectants or plant growth regulators have been attempted to increase the drought tolerance of plants (Seleiman et al., 2021). Among these, treatment using weak organic acids such as acetic acid is being studied in various plants due to its easy accessibility within the plant and nature-friendly characteristics (Mahmud et al., 2023). Rasheed et al. (2018) reported that upregulation of the acetic acid biosynthesis pathway in *Arabidopsis* can affect drought stress in plants. Based on this result it has been reported that acetic acid treatment can increase drought stress tolerance in various crop plants including corn and rice (Ogawa et al., 2021; Mahmud et al., 2023). Alfalfa (*Medicago sativa* L.) is a widely used forage crop with high feed value and good palatability (Kulkarni et al., 2018). Although many studies have been reported to increase the drought tolerance of alfalfa, no studies using acetic acid have been reported yet. This experiment was conducted to determine the effect of acetic acid treatment on drought tolerance in alfalfa.

Ⅱ. MATERIALS AND METHODS

1. Growth conditions and treatments

Alfalfa (cv. Vernal) seeds were sown in pot (height × diameter, 9 cm × 10 cm) filled with sand : horticultural medium (1:3, v/v) and then well watered. After 7 days, plant were thinned to 9 seedlings in each pot and fertilized every 3 day with ½ ×

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Hoagland solution (Phytotech Lab, USA) bottom watering. After 14 days, all pots were grouped into four groups, and treated with the control (½ × Hoagland, well-watering), AA (½ × Hoagland with 15 mM acetic acid, well-watering), D (½ × Hoagland, drought), and AA + D (½ × Hoagland with 15 mM acetic acid, drought) for 6 days. Acetic acid was treated every 2 days. After acetic acid treatment, all pots were exposed to drought stress for another 4 days. Plant were cultivated for 24 days at 23°C, humidity 55% and light/dark (16/8 h).

2. Measurement of plant relative water content (RWC)

Relative water content were calculated with the method described previously (Gao et al., 2019). Plant were weight separately (Fresh weight, FW), soaked in distilled water for half a day (Turgid fresh weight, TW), and then dried at 65°C for 3 days (Dry weight, DW). Relative water content (RWC) % = (Fresh weight − Dry weight) / (Total weight − Dry weight) × 100

3. Analysis of chlorophyll and carotenoid contents

Chlorophyll and carotenoid contents were calculated with the method described previously (Jeffrey and Humphrey, 1975). Briefly, 20 mg fresh leaf samples were immersed in 1 mL 95% (v/v) ethanol, extracted for 20 min. at 80°C. After cooling, absorbance was measured at 470 nm, 648 nm, and 664 nm using spectrophotometer (UV-1800, Shimadze Co., Kyoto, Japan).

Chl a = 13.36 × 664 nm - 5.19 × 648 nm

Chl b = 27.43 × 648 nm - 8.12 × 664 nm

Carotenoid = (1000 × 470 nm - 2.14 × Chl a - 97.64 × Chl b)/209

4. Hydrogen peroxide and proline analysis

Hydrogen peroxide (H_2O_2) was measured by the according to previously reported methods (Lee et al., 2022). Content of H_2O_2 was observed spectrophotometrically at 410 nm. Proline content was measured by the according to previous methods (Rahman et al., 2016).

5. Statistical analysis

Statistical analyses were performed using IBM SPSS statistics (IBM SPSS Statistics for windows, Version 27.0. Armonk, NY, USA) with independent samples t-test and significance testing at the 5% level.

Ⅲ. RESULTS AND DISCUSSION

The effects of various concentrations of acetic acid pretreatment on growth of alfalfa subjected to drought stress are shown in Fig. 1. In the control group, normal growth observed up to 15 mM acetic acid, and plant growth was inhibited at higher concentrations. Additionally, when exposed to drought stress after acetic acid pretreatment, 15 mM was the most effective, and higher or lower concentrations resulted in wilting. Therefore, subsequent acetic acid treatments were

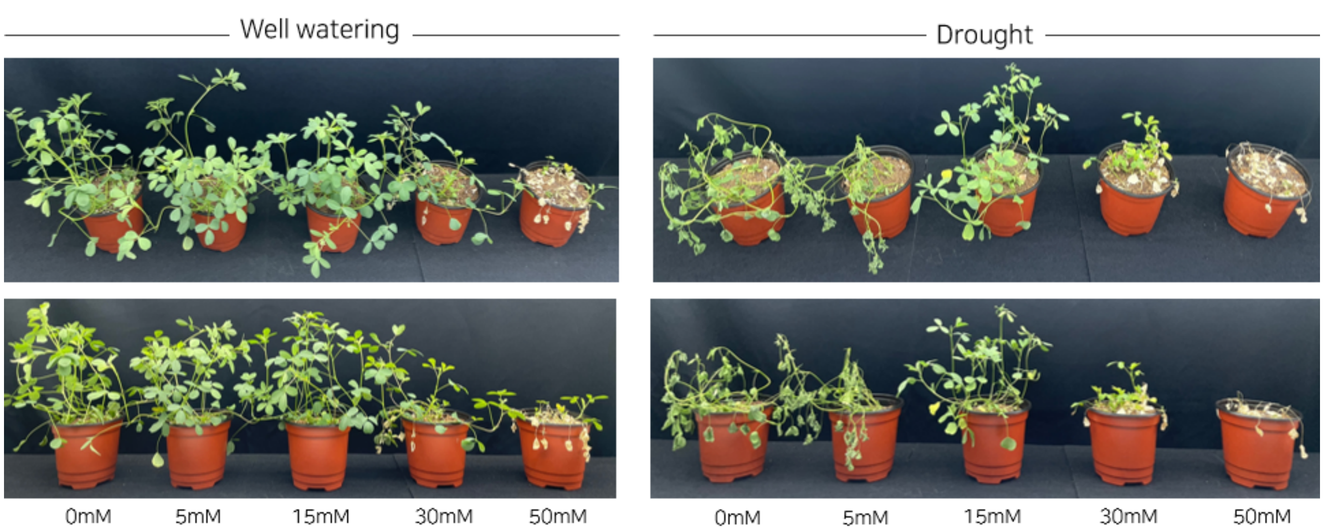

Fig. 1. Effects of different concentrations of acetic acid on alfalfa growth under drought stress.

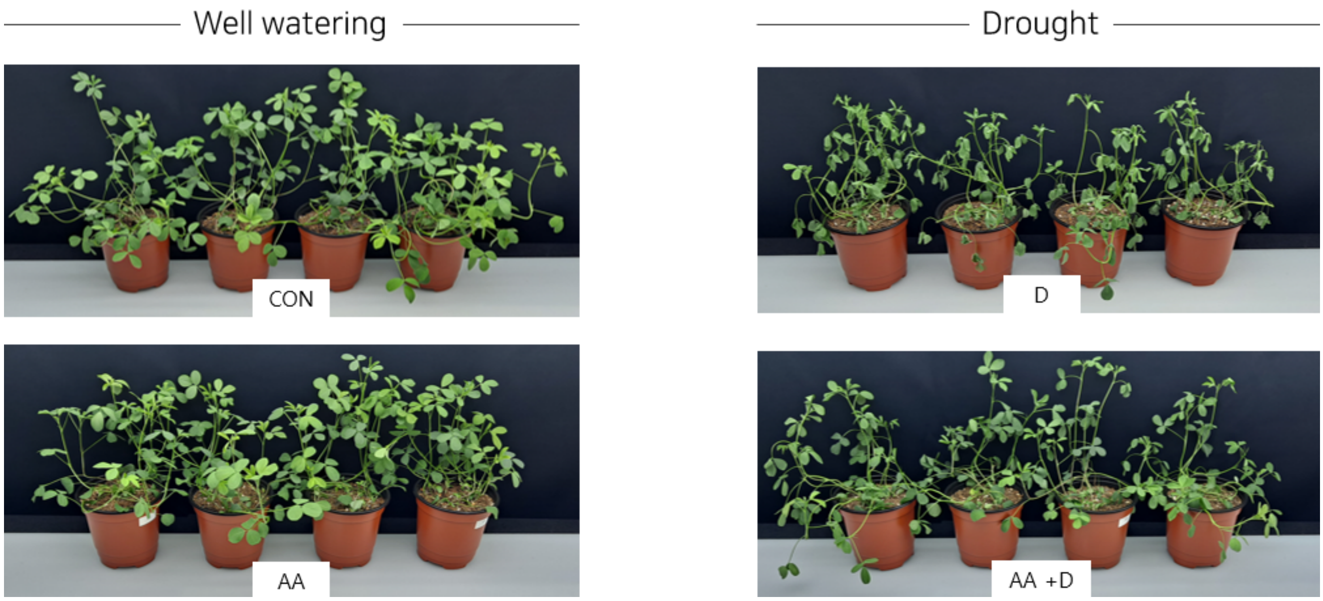

Fig. 2. Effect of optimal acetic acid concentration on the growth of alfalfa plants under drought stress. Alfalfa seedlings were treated with 15 mM of acetic acid for 6 days and exposed to drought stress.

performed at the same 15 mM.

The well-watered control group (CON) and the acetic acid pretreatment group (AA) did not show significant differences in the growth of alfalfa. While the treatment group (D) that was subjected to drought treatment for 6 days showed a wilted phenotype (Fig. 2). However, the drought stress treatment group after pretreatment with acetic acid (AA + D) did not show wilting and remained healthy (Fig. 2).

The growth of alfalfa plants was compared after acetic acid pretreatment. As a result, fresh weight, shoot length, and dry weight decreased by the 15 mM acetic acid treatment (AA) compared to the control group (Fig. 3). In the case of alfalfa that was only subjected to drought stress (D), fresh weight, dry weight, and shoot length all significantly decreased. However, in the drought treatment group after acetic acid pretreatment (AA + D), dry weight significantly decreased by 11%, but fresh weight significantly increased by 23%. On the other hand, there was no change in relative water contents (RWC) in the acetic acid pretreatment group that was not subjected to drought treatment. However, drought stress (D) dramatically decreased RWC, but in the drought treatment group after acetic acid pretreatment (AA + D), it increased by 28% and recovered similarly to the control level. These results suggest that the drought stress avoidance mechanism of alfalfa induced by acetic acid pretreatment is related to the maintenance of water content in the plant. Similar results were reported in cassava (Utsumi et al., 2019), where it was reported that when cassava was treated with acetic acid under drought stress conditions, RWC significantly increased compared to the untreated control. This suggests that pretreatment with acetic acid can protect plants from drought stress by maintaining water status on the plants.

Chlorophyll content was reduced by 21% in the acetic acid treatment (AA) compared to control (CON). Drought stress treatment (D) showed a significant increase of chlorophyll

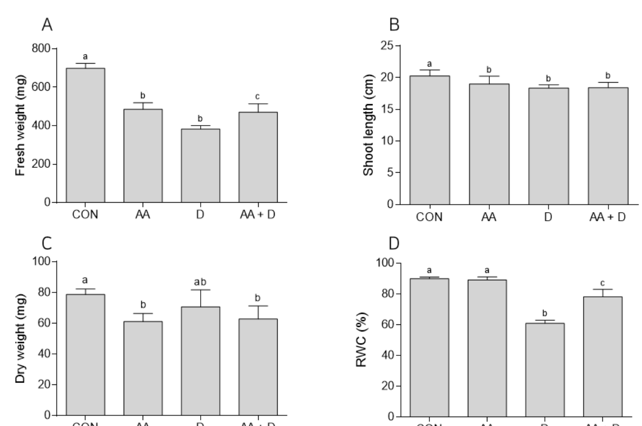

Fig. 3. Effect of acetic acid treatment on fresh weight, shoot length, dry weight, and relative water content (RWC) in alfalfa. Fresh weight of shoot (A) and shoot length (B), dry weight (C) and RWC (D).

compared to the control. But in the drought treatment after acetic acid pretreatment (AA + D), it increased by 13% compared to D (Fig. 4A). Similarly carotenoid content decreased by 7% in AA and increased in D group compared to the CON group, and not changed in AA + D group (Fig. 4B). Drought stress is known to reduce chlorophyll content (Batra et al., 2014). However, Agathokleous et al. (2020) reported that when plants are directly exposed to high levels of stress, chlorophyll concentration decreases, but under low levels of stress, they cope with stress by regulating chlorophyll levels.

The results of the experiment showed that chlorophyll and carotenoid content increased in drought conditions compared to control and significantly decreased in acetic acid-treated plants. This suggests that the decrease in chlorophyll and carotenoid contents by acetic acid treatment may be due to a decrease in stress levels by activating the function of acetic acid.

In plants, proline is produced and accumulated to stabilize intracellular structures by producing and accumulating cellular osmolytes (Jung et al., 2010) and is accumulated to cope with various environmental stresses (Wang et al., 2022). The proline content increased significantly in the drought environment compared to control and decreased significantly in acetic acid-treated plants (Fig. 5). This suggests that the acetic acid treatment helped alleviate the stress experienced by the plants. Interestingly the acetic acid treatment alone without the drought treatment also resulted in an increase of proline levels. This can be inferred that acetic acid positively induces proline accumulation in plants. Mahmud et al. (2023) also reported that acetic acid treatment in maize increased proline accumulation by upregulating proline-expressing genes. Due to the different gene expression mechanisms in different plants, further experiments is necessary to explore the mechanism of proline accumulation in alfalfa.

Hydrogen peroxide content showed no significant difference between control (CON) and acetic acid treated (AA). Drought treatment (D) increased by 78% compared to the control. Under the same drought conditions, the acetic acid treatment (AA + D) showed a 20% decrease compared to D (Fig. 6). Plants exposed to drought stress accumulate reactive oxygen species (ROS) such as superoxide and hydrogen peroxide and cause exhibit oxidative stress (Goldack et al., 2014). Hydrogen peroxide is one of ROS characterized by low reactivity (Huang et al., 2019). The increase of H_2O_2 under drought stress was

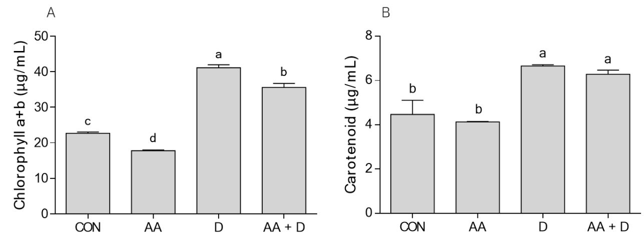

Fig. 4. Effect of acetic acid treatment on chlorophyll and carotenoid contents in alfalfa.

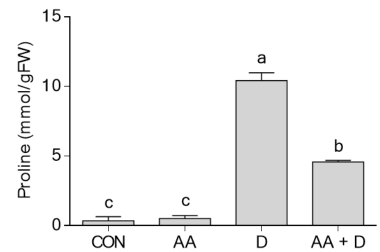

Fig. 5. Effect of acetic acid treatment on proline contents in alfalfa.

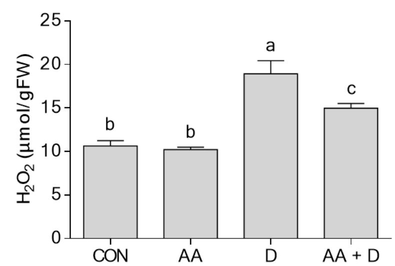

Fig. 6. Effect of acetic acid treatment on H_2O_2 contents in alfalfa.

significantly reduced after acetic acid treatment (Fig. 6). This suggests that acetic acid can help alleviate drought-induced oxidative stress.

Ⅳ. CONCLUSIONS

This study shows that exogenous application of acetic acid in alfalfa can alleviate drought stress in plant. The signaling effects of acetic acid on the metabolism of alfalfa need to be studied more specifically but the results of this study show that acetic acid will play positive role against drought stress in alfalfa. Further research is needed to investigate the function of acetic acid in relation to drought stress at the molecular levels in alfalfa.

V. ACKNOWLEDGMENTS

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