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Effects of Drought Stress on C4 Photosynthetic Enzymes and $ $13C Values in Leaves of the C3 Plant Salsola juncea (Postprint)

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Abstract

This study investigated the representative C3 desert subshrub Salsola junatovii Botsch. from the Chenopodiaceae family. A pot-controlled water experiment was conducted with normal water and three drought stress treatments (mild, moderate, and severe) to measure leaf water content, activities and protein expression levels of four C4 photosynthetic enzymes [phosphoenolpyruvate carboxylase (PEPC), NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PEPCK)], and stable carbon isotope ratios ($ $13C values) under different drought stress levels. Additionally, $ $13C values of leaves from six field sites with different mean annual precipitation were determined to clarify the effects of drought stress on C4 photosynthetic enzymes and $ $13C values in S. junatovii, and to explore whether this species adapts to drought stress by enhancing C4 pathway expression. The results showed: (1) Leaf water content decreased with increasing drought stress severity; (2) Among the four photosynthetic enzymes, only PEPC and NAD-ME were affected by drought stress. The activity and protein expression levels of these two enzymes showed consistent trends, increasing initially with intensifying drought stress, reaching maximum values under moderate drought stress, and then decreasing sharply under severe drought stress. NADP-ME activity, PEPCK activity, and their protein expression levels were not affected by drought stress; (3) Although $ $13C values of S. junatovii leaves increased under mild and moderate drought stress, peaking under moderate drought stress, they remained within the C3 plant range under all stress treatments. $ $13C values of field samples indicated that samples from the three regions with relatively low annual precipitation had higher $ $13C values.

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Full Text

Preamble

Changes of C4 Photosynthetic Enzymes and $ $13C Values of C3 Desert Plant *Salsola junatovii* Botsch. Under Soil Drought Stress

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Abstract

Salsola junatovii Botsch., a representative C3 desert subshrub in the genus *Salsola* (Chenopodiaceae), was subjected to a pot-controlled water experiment with four treatments: normal water conditions and mild, moderate, and severe drought stress. We measured leaf water content, activities and protein expression levels of four C4 photosynthetic enzymes [phosphoenolpyruvate carboxylase (PEPC), NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PEPCK)], and stable carbon isotope ratios ($ $13C values) under different drought intensities. Additionally, we determined leaf $ $13C values from six field sites with varying mean annual precipitation to clarify how different drought stresses affect C4 photosynthetic enzymes and $ $13C values in *S. junatovii*, and to explore whether this C3 plant enhances C4 pathway expression to adapt to drought. The results showed that: (1) leaf water content decreased with increasing drought stress; (2) among the four photosynthetic enzymes, only PEPC and NAD-ME were affected by drought stress, with both enzyme activities and protein expression levels showing similar trends—increasing with drought intensity, reaching maximum values under moderate stress, then declining sharply under severe stress; NADP-ME activity and PEPCK activity and protein expression were unaffected by drought; (3) although leaf $ $13C values increased under mild and moderate drought, peaking under moderate stress, they remained within the C3 plant range; field samples from areas with relatively low annual precipitation showed higher $ $13C values.

Keywords: photosynthetic carbon assimilation pathway; C3 desert plant; drought stress; C4 photosynthetic enzymes; *Salsola junatovii*

Introduction

The photosynthetic carbon assimilation pathways of angiosperms can be classified into three types based on differences in initial products and carbon metabolism during CO2 assimilation: C3, C4, and crassulacean acid metabolism (CAM) pathways, with corresponding plants designated as C3, C4, and CAM plants [1]. The C4 pathway is believed to have evolved independently

from the C3 pathway more than 60 times [2-3], with C4 photosynthesis evolving independently in each family and genus of angiosperms [4]. This polyphyletic evolutionary pattern suggests that the transition from C3 to C4 photosynthesis is relatively simple [5]. Any environmental factor that reduces atmospheric CO2 concentration and enhances photorespiration can induce the emergence of C4 photosynthetic pathways [3]. The shift from C3 to C4 photosynthesis represents both an adaptive evolutionary response to stress and a strategy for enhancing survival and competitive ability [6]. Compared with C3 plants, C4 plants possess a CO2-concentrating mechanism that confers higher photosynthetic efficiency and water use efficiency under high temperature, strong light, and low water conditions [7], giving them a competitive advantage over C3 plants in such environments [1].

While most plants utilize only one photosynthetic carbon assimilation pathway [1], these pathways can change under specific environmental conditions [8]. Among various environmental regulations, drought-induced transitions between C3 and C4 pathways are the most common [8-12], manifesting in photosynthetic structure, physiological, and biochemical characteristics. For example, the amphibious sedge *Eleocharis vivipara* exhibits Kranz anatomy and high activities of C4 photosynthetic enzymes (PEPC, pyruvate phosphate dikinase (PPDK), and NAD-ME) under terrestrial conditions, but lacks Kranz anatomy and shows low C4 enzyme activity under submerged conditions [10]. The grass *Alloteropsis semialata* fixes CO2 via the C3 pathway in cool, rainy regions but via the C4 pathway in hot, arid regions [1]. The C3 plant *Phragmites australis* shifts its photosynthetic carbon assimilation pathway from C3 toward C4 with increasing soil water stress, exhibiting C3-like photosynthesis in marsh environments, C3-C4 intermediate (C3-biased) photosynthesis in saline meadows, and C3-C4 intermediate (C4-biased) photosynthesis in dune environments [11, 13]. In the desert C3 plant *Hedysarum scoparium*, activities of C4 photosynthetic enzymes (PEPC and NAD-ME) gradually increase with water stress, working synergistically with the antioxidant protection enzyme system to resist intensifying drought [14]. Increasing drought stress enhances the possibility of carbon assimilation pathway transition from C3 to C4, allowing plants to improve water use efficiency through a series of changes in leaf anatomy, ultrastructure, and physiological-biochemical characteristics [15-18]. Thus, examining photosynthetic carbon assimilation pathway transitions provides an important perspective for analyzing how plants adapt to arid environments.

Plant photosynthetic carbon assimilation pathways are easily influenced by environmental factors and exhibit environmental plasticity, making them closely related to global climate change and human activities [19]. Under global climate change, whether C3 plants will undergo adaptive adjustments toward C4 photosynthesis to better survive and fulfill their ecological roles represents a crucial question regarding plant adaptation to environmental change. Arid regions are recognized as being among the most sensitive to global change [20], with water serving as the primary driver of ecosystem processes and playing a vital role in plant growth and development [21-22]. Consequently, plant biological responses

in these regions are mainly regulated by water availability, making them ideal materials for studying plant adaptation to global change.

Salsola junatovii Botsch., belonging to the genus *Salsola* (Chenopodiaceae), is endemic to China [23] and is a semi-shrub typical of C3 desert plants. Its stable carbon isotope ratio ($ $13C value) is -20.4‰ [24], which falls within the $ $13C range of C3 plants (-30‰ to -21‰) [25]. By investigating the activities and protein expression levels of key C4 photosynthetic enzymes (PEPC, NAD-ME, NADP-ME, and PEPCK) and $ $13C values in *S. junatovii* under drought stress, this study addresses two questions: (1) How do different drought stresses affect C4 photosynthetic enzymes and $ $13C values in *S. junatovii*? (2) Does this C3 plant adapt to drought stress by enhancing C4 photosynthetic pathway expression? The expected results will contribute to understanding the adaptive strategies of desert C3 plants under different environmental conditions and are significant for 深入研究干旱区植物响应环境因子及其适应机制.

1 Materials and Methods

1.1 Plant Materials

Seeds of *S. junatovii* were collected from Kuqa, Xinjiang in October 2015 and stored at 4°C. The water control experiment was conducted at the Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences in 2017. Seeds were sown outdoors in April 2017 in plastic pots (30 cm height × 26 cm inner diameter) filled with a 3:1 (v:v) mixture of substrate soil and sandy soil with a field capacity of 24.5%. Plants received natural light and were covered with a rain shelter during the experiment, with normal water management maintained. One week before formal drought treatment, seedlings were thinned to three plants per pot. Drought stress was applied to 12-week-old plants.

The drought stress experimental design followed Wen et al. [26] and included four treatments: control (CK, 80% field capacity), mild drought stress (T1, 60% field capacity), moderate drought stress (T2, 45% field capacity), and severe drought stress (T3, 35% field capacity), with four biological replicates per treatment. During the formal treatment period, lost water was replenished daily using the weighing method to maintain the specified conditions. After 5 days of treatment, leaves were collected at 11:00 on August 3. Some leaves were used for immediate water content determination in the laboratory; some CK leaves were fixed directly in FAA (formalin:acetic acid:alcohol = 18:1:1) for anatomical observation; some leaves were dried in silica gel, oven-dried, and ground for $ $13C analysis; remaining leaves were immediately frozen in liquid nitrogen for enzyme activity and protein expression assays. Cotyledons were also collected during plant growth, with fully expanded 10-12-day-old cotyledons fixed in FAA for anatomical observation.

Field samples of *S. junatovii* were collected from six sites in Xinjiang (Hejing [YQ], Akto [AKT], Kuqa [KC], Baicheng [BC], Wuqia [WQ], and Akqi [AHQ]) selected based on mean annual precipitation data from the China Meteorolog-

ical Administration and WorldClim. Collected samples were dried in silica gel for subsequent $ $13C determination. Geographic information and mean annual precipitation for the five sampling sites are presented in Table 1 . Plant materials were identified by the authors, with voucher specimens deposited at the Herbarium of Xinjiang Institute of Ecology and Geography (XJBI).

1.2 Experimental Methods

1.2.1 Anatomical Structure of Cotyledons and Leaves FAA-fixed cotyledons and true leaves were dehydrated through an ethanol series, cleared with ethanol-xylene, infiltrated with xylene-paraffin, and embedded. Embedded materials were sectioned at 8 m thickness using a rotary microtome (Leica 2235), double-stained with safranin and fast green, cleared with xylene, and mounted with neutral balsam to prepare permanent slides [27]. Sections were observed and photographed under a light microscope (Olympus DP70) with three replicates each for cotyledons and true leaves.

1.2.2 Leaf Water Content Approximately 2 g of fresh leaves were weighed and oven-dried at 105°C for 2 h, then at 80°C for 8-10 h to constant weight before measuring dry weight. Leaf water content was calculated as: (fresh weight - dry weight) / fresh weight × 100%. Measurements were repeated three times.

1.2.3 Leaf C4 Photosynthetic Enzyme Activities Enzyme extraction followed Voznesenskaya et al. [28] with slight modifications. Fresh leaf tissue (0.3 g) was ground with a small amount of quartz sand and 2.0 mL ice-cold 100 mmol·L^{-1} Tris-HCl buffer (pH 7.8 containing 5% glycerol, 1% polyvinylpyrrolidone [PVP], 1 mmol·L^{-1} EDTA, and 10 mmol·L^{-1} mercaptoethanol) in an ice bath. The homogenate was centrifuged at 15,000 g for 15 min at 4°C, and the supernatant was used for assays. PEPC, NAD-ME, and NADP-ME activities were measured following Wen et al. [29], while PEPCK activity was determined following Wang et al. [30]. All assays were repeated three times.

1.2.4 Protein Expression Levels of C4 Photosynthetic Enzymes Leaf protein extraction followed Wang et al. [30]. Leaf tissue (0.1 g) was ground to powder in liquid nitrogen in a pre-chilled mortar, transferred to a centrifuge tube containing 1 mL protein extraction buffer, mixed thoroughly, and centrifuged at 15,000 g for 15 min at 4°C. The supernatant was mixed with protein loading buffer and boiled for 5-10 min. Subsequent electrophoresis, transfer, blocking, and primary and secondary antibody incubation steps followed Wen et al. [29]. Images were captured after exposure with ECL luminescent solution and quantified using Image J software, with the control group set to 100%. All measurements were repeated three times.

1.2.5 Leaf $ $13C Values Leaf $ $13C values were determined using a stable gas isotope mass spectrometer (Delta V Advantage). Each sample was prepared

in triplicate. $ $13C (‰) was calculated as: [(Rsample - Rstandard) / Rstandard] × 10^3, where Rsample is the isotopic abundance ratio of the sample and Rstandard is that of the standard [31-32].

1.3 Data Processing

Data among different treatments were analyzed using one-way ANOVA and multiple comparisons (LSD) with SPSS 22.0.

2 Results

2.1 Anatomical Structure of Cotyledons and Leaves in *S. junatovii*

The anatomical structures of cotyledons and true leaves in *S. junatovii* were similar (Fig. 1 [Figure 1: see original paper]), both exhibiting typical C3 characteristics: absence of Kranz anatomy, presence of epidermis, 2-3 layers of palisade cells, and discontinuous bundle sheath cells. The main difference was that true leaves had larger water storage cells compared with cotyledons. Combined with the $ $13C values, these anatomical features confirm that *S. junatovii* is a C3 plant.

2.2 Effects of Drought Stress on Leaf Water Content

Leaf water content in *S. junatovii* decreased gradually with intensifying drought stress (Fig. 2 [Figure 2: see original paper]). Compared with the control, leaf water content was less affected under mild drought stress but decreased by 14.6% and 37.7% under moderate and severe drought stress, respectively ($P < 0.05$).

2.3 Effects of Drought Stress on Leaf C4 Photosynthetic Enzyme Activities

PEPC activity increased initially then decreased with intensifying drought stress, with significant differences between the control and all stress treatments (Fig. 3 [Figure 3: see original paper]). Compared with the control, PEPC activity increased significantly by 44.6% and 54.5% under mild and moderate drought stress, respectively, but decreased significantly by 33.1% under severe drought stress ($P < 0.05$). NADP-ME activity was very low in the control (0.196 $\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}\text{Chl}$) and was not significantly affected by drought stress, with no significant differences among treatments ($P > 0.05$). NAD-ME activity increased initially then decreased with intensifying drought stress, reaching its maximum under moderate drought stress (0.754 $\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}\text{Chl}$) before declining sharply under severe stress. Compared with the control, NAD-ME activity increased significantly by 24.5% and 61.6% under mild and moderate drought stress, respectively, and decreased significantly by 37.5% under severe drought stress ($P < 0.05$). PEPCK activity was not significantly affected by drought stress, with no significant differences among treatments ($P > 0.05$).

2.4 Effects of Drought Stress on Protein Expression Levels of Leaf C4 Photosynthetic Enzymes

PEPC protein expression increased initially then decreased with intensifying drought stress (Fig. 4 [Figure 4: see original paper]). Compared with the control, PEPC protein expression increased significantly by 7.6% and 14.3% under mild and moderate drought stress, respectively ($P < 0.05$), but decreased under severe drought stress without significant difference from the control ($P > 0.05$). NADP-ME was not detected in *S. junatovii*. NAD-ME protein expression increased initially then decreased with intensifying drought stress, reaching its maximum under moderate drought stress before declining sharply under severe stress. Compared with the control, NAD-ME protein expression increased significantly by 9.7% and 35.8% under mild and moderate drought stress, respectively, and decreased significantly by 7.4% under severe drought stress ($P < 0.05$). PEPCK protein expression was not significantly affected by drought stress, with no significant differences among treatments ($P > 0.05$).

2.5 Effects of Drought Stress on Leaf $ $13C Values

Leaf $ $13C values decreased slowly with increasing drought stress, reaching their lowest value (-23.35‰) under moderate drought stress (Fig. 5 [Figure 5: see original paper]). $ $13C values under mild and severe drought stress did not differ significantly from the control ($P > 0.05$), but the value under moderate drought stress differed significantly from other treatments ($P < 0.05$).

$ $13C values of the six field samples showed that samples from YQ, AKT, and KC (areas with relatively low annual precipitation) ranged from -21.57‰ to -20.97‰, with no significant differences among these three sites ($P > 0.05$). Samples from BC, WQ, and AHQ (areas with relatively high annual precipitation) ranged from -24.82‰ to -24.02‰, also with no significant differences among them ($P > 0.05$). However, $ $13C values differed significantly between the two groups with substantially different mean annual precipitation ($P < 0.05$) (Table 1).

3 Discussion

PEPC, NADP-ME, NAD-ME, and PEPCK are key photosynthetic enzymes in the C4 pathway. In C4 photosynthesis, CO2 entering mesophyll cells is first hydrated to HCO_3^- in the cytoplasm, then catalyzed by PEPC to form oxaloacetate. Based on the decarboxylase involved, C4 pathways are classified into three types: NADP-ME, NAD-ME, and PEPCK types, with NADP-ME, NAD-ME, and PEPCK serving as the respective decarboxylases [1]. In this study, only PEPC and NAD-ME were affected by drought stress. PEPC activity in *S. junatovii* increased gradually with drought intensity, peaked under moderate drought stress, then declined sharply under severe stress (Fig. 3). Under mild and moderate drought stress, changes in PEPC protein expression paralleled those in enzyme activity, while under severe drought stress, protein

expression decreased but did not differ significantly from the control ($P > 0.05$) (Fig. 4). The increased PEPC activity under mild and moderate drought stress may partly result from increased PEPC protein synthesis, although elevated PEPC gene transcription or protein phosphorylation levels can also enhance PEPC activity [29, 33]. Both NAD-ME activity and protein content were affected by drought stress, showing consistent trends: increasing initially then decreasing with intensifying drought stress, peaking under moderate drought stress, then declining sharply under severe stress (Fig. 3 and Fig. 4). In the desert C3 plant *Hedysarum scoparium*, PEPC and NAD-ME activities also increased gradually with water stress [14]. In another desert C3 plant, *Salsola abrotanoides*, PEPC activity, gene expression, and protein expression increased with drought stress, NADP-ME activity increased, while NAD-ME activity and expression decreased [12]. Although the specific enzymes affected by drought stress vary among species, drought consistently enhances PEPC activity in C3 plants to some degree. C3 plants contain C4-type PEPC isozymes [34-35] that participate in CO2 fixation [36]; thus, increased PEPC activity can enhance CO2 fixation via PEPC, potentially strengthening the C4 pathway in C3 plants. In this study, PEPCK activity and protein content in *S. junatovii* were unaffected by drought stress (Fig. 3 and Fig. 4). Similarly, in the desert C3 plant *S. abrotanoides*, PEPCK protein content was unaffected by drought [12] (though PEPCK activity was not measured). Interestingly, under normal water conditions, PEPCK activity in *S. junatovii* was 2.3 $\mathrm{mol \cdot min^{-1} \cdot mg^{-1}Chl}$, similar to that in the C4 species *Salsola arbuscula* (2.5 $\mathrm{mol \cdot min^{-1} \cdot mg^{-1}Chl}$, unpublished data), suggesting that PEPCK may have functions beyond its role in the C4 cycle.

Leaf $ $13C values are commonly used as a relatively rapid indicator of plant photosynthetic type. C3 plants typically have $ $13C values of -30‰ to -21‰, while C4 plants range from -15‰ to -10‰ [25]. This difference arises because C3 and C4 plants discriminate against CO2 differently during photosynthesis, leading to distinct carbon isotope fractionation patterns and stable carbon isotope compositions [37-38]. In this study, leaf $ $13C values in *S. junatovii* increased under mild and moderate drought stress compared with the control (Fig. 5), likely because elevated PEPC and NAD-ME activities and protein contents (Fig. 3 and Fig. 4) strengthened the C4 pathway, and as C4 pathway contribution increased, carbon isotope fractionation was affected, causing $ $13C values to trend upward. However, under the drought stresses examined, the C3 pathway still dominated in *S. junatovii*, with $ $13C values remaining within the C3 plant range. $ $13C values are positively correlated with plant water use efficiency and can reflect water use efficiency levels [12, 37, 39]. Therefore, the C3 plant *S. junatovii* improved water use efficiency by developing the C4 pathway under mild and moderate drought stress. Field sample $ $13C values showed that samples from areas with lower annual precipitation (YQ, AKT, and KC) had higher $ $13C values than those from areas with higher precipitation (BC, WQ, and AHQ) (Table 1), indicating that at a longer temporal scale, *S. junatovii* in drier regions has higher leaf water use efficiency.

4 References

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