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Physiological and metabolic changes in Selaginella tamariscina in response to desiccation and resurrection

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Research Article

Keywords: antioxidant defense, dehydration, osmoregulation, organic metabolite, oxidative damage, photochemical efficiency

Posted Date: September 15th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3321347/v1

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Version of Record: A version of this preprint was published at Plant Growth Regulation on November 4th, 2024. See the published version at https://doi.org/10.1007/s10725-024-01237-8.

Abstract

As a typical resurrection plant, *Selaginella tamariscina* exhibits remarkable drought tolerance and resurrection capacity. However, the mechanism of resurrection associated with the alteration of global organic metabolites in *S. tamariscina* has not been fully elucidated. Objectives of the study were to investigate drought tolerance and recovery capacity of *S. tamariscina* based on physiological response and to further reveal potential mechanism of resurrection related to changes in antioxidant defense and differential metabolites under drought stress and after rehydration. Results showed that severe drought reduced leaf relative water content from 90–18%, resulting in extreme declines in chlorophyll content and photochemical efficiency as well as a significant increase in malondialdehyde content in leaves, but *S. tamariscina* plants could rapidly recover within 3 days of rehydration. Superoxide dismutase, peroxidase, and catalase were significantly activated by dehydration and rehydration. In addition, drought-induced accumulations of citric acid and ribitol could be maintained at higher levels in response to rehydration. Although most of organic metabolites were not affected significantly by dehydration (lactic acid, ribonic acid, arabinitol, and erythritol) or decreased sharply under drought stress (glycine, alanine, γ-aminobutyric acid, proline, glyceric acid, vanillic acid, arabinose, and rhamnose), but *S. tamariscia* has the ability to quickly recover or increase the contents of these organic metabolite after rehydration. Current findings indicated that enhanced antioxidant defense system could be one of the main pathways for acquisition of desiccation tolerance and resurrection capacity, thereby alleviating oxidative damage to *S. tamariscina* plants. The accumulation of various organic metabolites played critical roles in underlying mechanism of resurrection due to their positive function associated with osmotic adjustment, osmoprotection, antioxidant, and energy metabolism.

Introduction

Plants often suffer from a variety of abiotic stresses in nature, including drought, salinity, high temperature, heavy metal pollution etc., among which drought is considered to be one of the most significant threats to global agricultural systems (Zhang et al. 2022). With the aggravation of global warming, the increasing occurrence of extreme weather events is sweeping across the globe leading to reduced availability of water resources for agriculture and horticulture (Ostberg et al. 2018). Drought stress impedes plant growth and development by affecting different biochemical and physiological mechanisms such as photosynthesis, antioxidant systems, and hormone signaling (Yang et al. 2021). During a long-term adaptation to drought condition, plants have developed four main mechanisms including drought escape, drought avoidance, drought tolerance, and drought recovery. Drought avoidance and drought tolerance are usually called drought resistance (Fang and Xiong 2015). Plants often experience multiple cycles of drought, rewatering, and re-drought due to complex and ever-changing environments. Therefore, it is important not only to focus on the drought tolerance in plants but also to consider their ability to recover from drought stress, which is crucial for predicting the fate of natural vegetation under extreme climate conditions, improving crop management, and guiding agricultural breeding efforts.

Selaginella tamariscina is a typical resurrection plant with remarkable drought tolerance and resurrection capacity. It not only has ornamental value for landscaping, but also exhibits medicinal value as a traditional perennial Chinese medicine for the treatment of cancer, hepatitis, and diabetes (Bailly 2021; Setyawan 2009; Shim et al. 2018). *S. tamariscina* can survive under extreme drought stress, even when all leaves dehydrate to fully curl up and turn yellow. However, its leaves quickly regain green color and unfold when being exposed to water, earning a popular nickname “Plant with Nine Lives” in folklore (Challabathula and Bartels 2013). Possible mechanism of desiccation tolerance and resurrection in the *S. tamariscina* has been reported. For example, the study of Agduma and Sese demonstrated that the accumulation of sugar could exhibit a critical role in osmotic adjustment (OA) when *S. tamariscina* responded to dehydration and rehydration, but proline only significantly increased under desiccation stress (Agduma and Sese 2016). Proteomic analysis identified many desiccation-responsive proteins in *S. tamariscina* associated with photosynthesis, antioxidant defense, and translational modification (Wang et al. 2010). Similar findings were showed in the study of Gu et al. who found that multiple genes involved in antioxidant defense system, polyamine and carbohydrate metabolism, and stress-defensive protein biosynthesis such as dehydrins and aquaporins could contribute to desiccation tolerance of *S. tamariscina* due to beneficial roles of these metabolites and proteins in antioxidant, OA, and signal transduction (Gu et al. 2019). In addition, abscisic acid signaling was one of important desiccation tolerance strategies in *S. tamariscina* (Liu et al. 2008; Xu et al. 2018).

Water deficit disrupts reactive oxygen species (ROS) homeostasis leading to oxidative stress, which severely impairs plant growth and survival. Antioxidant defense system scavenges over-generation of ROS to detoxify oxidative damage to plant cells (Hasanuzzaman et al. 2013). Additionally, abundant organic metabolites within plant cells play crucial roles in signal transduction, OA, osmoprotection, energy supply, and free radical scavenging (Sharma et al. 2019). Metabolomics focuses on extensive metabolites associated with the comprehensive metabolic profiling under specific condition, exhibiting significant importance in studying global regulatory systems in plants under stressful conditions (Kumar et al. 2021). Previous studies have identified many important metabolites from *S. tamariscina* such as amentoflavone, isocryptomerin, bioflavonoid, and flavonoid with pharmacological functions of antibiosis, anti-inflammation, and antioxidant (Lee et al. 2009; Jung et al. 2006; Shim et al. 2018). Limited information has been reported to reveal mechanism of resurrection in *S. tamariscina* associated with the alteration of global metabolites profiling. Current study investigated drought tolerance and recovery capacity of *S. tamariscina* based on physiological response and further revealed potential mechanism of resurrection related to dynamic changes in antioxidant defense and differential metabolites in leaves during drought and recovery phases. The findings will help to better understand mechanism of resurrection in plants and provide important background information for crop genetic improvement in drought recovery.

Materials and methods

Test materials

S. tamariscina plants were collected from germplasm resource nursery which was located in Sichuan Agricultural University, Chengdu, Sichuan. These plants were propagated through pot cultivation in the greenhouse. The plants were grown in pots which was filled with sandy loam soil as growth medium and watered by Hoagland's solution once a week (Hoagland and Arnon 1950). After one month of establishment in the greenhouse, the plants with same size were chosen as experimental materials, and then were removed to controlled growth chambers for 7 days of acclimation (26/22°C, 750 μmol•m^{-2}•s^{-1} PAR, and 65% humidity). Leaf samples were taken as the well-watered control. After that, the irrigation was stopped for 40 days as drought treatment when leaves completely curled due to water deficit. All plants were re-watered for 3 days as the rehydration treatment when curled leaves were fully unfolded and regained green color. Each treatment in the experimental included six independent biological replications (6 pots) and each pot had one plant.

Measurement of physiologic parameters

Leaf relative water content (RWC) was calculated using the formula RWC = (FW-DW) / (TW-DW) × 100%. FW indicated fresh weight of fresh leaves. TW indicated turgid weight (fresh leaves were immersed in distilled water for 12 hours at 4°C. After the leaves were fully saturated, they were removed from the water and the surface moisture was gently wiped off. Turgid leaves were then weighed). DW indicated dry weight (turgid leaves were placed in a forced-air drying oven at 105℃ for 15 minutes, followed by drying at 75℃ until a constant weight was achieved) (Barrs and Weatherley 1962). For determination of chlorophyll (Chl) content, a total of 0.1 g fresh leaves were soaked in 10 ml of dimethyl sulfoxide (DMSO) solution and then placed in the dark until all leaves turned into white. Absorbance of extracts was detected at 663 and 645 nm, respectively (Arnon 1949). Chl fluorescence parameters including maximum quantum yield of PSII photochemistry (Fv/Fm) and performance index on absorption basis (PI_{ABS}) were determined using the Chl fluorescence system (Pocket PEA, Hansatech, United Kingdom) (Zeng et al. 2021).

For determinations of MDA content and three antioxidant enzyme activities including superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), a 0.1 g of fresh leaves were ground with 1.5 m of pre-cooled 150 mM phosphate buffer and then centrifuged at 12000 g for 20 min at 4℃ to get the supernatant. For MDA content, a 0.5 ml of the supernatant was mixed with 1 ml of 20% trichloroacetic acid containing 0.5% thiobarbituric acid, and then put in water bath at 95℃ for 15 min. After being centrifuged at 12000 g for 10 min, the absorbance of supernatant was measured at 532 and 600 nm (Dhindsa et al. 1981). For CAT activity, a 50 μl of the supernatant was added into 0.95 ml of 50 mM phosphate buffer, and then mixed with 0.5 ml of 45 mM H_2O_2. The absorbance of mixture was measured at 240 nm (Maehly and Chance 1954). For POD activity, a 25 μl of the supernatant was mixed with 925 μl of 100 mM HAc-NaAc buffer and 0.5 ml of 0.25% guaiacol, and then a 50 μl of 0.75% H_2O_2 was added to start the reaction. The absorbance of mixture was measured at 470 nm (Maehly and Chance 1954). The SOD activity was detected by using the method of nitrogen blue tetrazolium (Giannopolitis and Ries 1977).

Metabolome determination

Metabolite extraction procedure was based on the method of Li et al. (Li et al. 2020). Processed samples were analyzed using combined two-dimensional gas chromatography/time of flight mass spectrometry (GC-TOFMS, Pegasus 4D, LECO Corporation, St. Joseph, MI, USA). The GC-TOFMS analysis procedure was referenced from the study of Li et al. (Li et al. 2020). Metabolites were identified by using Chroma TOF software (v. 4.50.8.0, LECO, St. Joseph, MI, USA) and commercially available compound libraries: NIST 2005 (PerkinElmer Inc., Waltham, MS), Wiley 7.0 (John Wiley and Sons Ltd. Hoboken, NJ). Relative retention times and mass to charge (m/z) ratios which were used for identification and quantification of 33 metabolites in leaves of *S. tamariscina* was recorded in Table S1.

Data analysis

The data were recorded and organized by using a Microsoft Excel 2016 software, and the analysis of variance (ANOVA) and significance testing ($P < 0.05$) were performed using a SPSS 27.0 software. Graphs were generated using the Origin 2021 software.

Results

Effects of drought and rehydration on leaf water status and photochemical parameters

As shown in Fig. 1A, leaves were erect, well-expanded, and had a fresh green color under normal water condition. After 40 days of drought stress, all leaves curled inward, shrank, and turned yellow. Leaves unfolded again and the leaf color re-greened after 3 days of rehydration. RWC in leaves maintained at 90% under normal water condition. After 40 days of drought treatment, leaf RWC decreased to 18%. However, the RWC significantly increased and reached to 89% following 3 days of rehydration (Fig. 1B). Drought stress induced significant declines in Chl content, Fv/Fm, and PI_{ABS}, whereas these parameters significantly recovered after 3 days of rehydration (Fig. 1C).

Effects of drought and rehydration on oxidative damage and antioxidant enzyme activities

SOD activity significantly increased after 40 days of drought stress, and then slightly decreased after 3 days of rehydration, but the decrease was not significant (Fig. 2A). CAT activity exhibited a similar trend with the change in SOD activity in response to drought and rewatering (Fig. 2B). Dehydration induced a significant increase in CAT activity which was also maintained at significantly higher level after 3 days of recovery (Fig. 2B). POD activity significantly increased after drought stress and reached to the highest level at 3 days of recovery (Fig. 2C). Dehydration led to a sharp increase in MDA content in leaves (Fig. 2D). As compared to drought stress, the MDA content decreased significantly after 3 days of rehydration, but it was still significantly higher than that in well-watered plants (Fig. 2D).

Change in metabolites profiling in response to drought and rehydration

A total of 33 differential metabolites in leaves were detected in response to drought and rewatering. These metabolites included 11 amino acids, 11 organic acids, and 11 other metabolites (Fig. 3A and B). PCA showed that the first or second principal component accounted for 33.8% or 40.7% of the variance, respectively (Fig. 3A). There were significant differences in 33 metabolite levels in response to dehydration and rehydration based on PCA and heat map (Fig. 3A and B). Under drought, the contents of total metabolites, amino acids, organic acids, and other metabolites in leaves significantly decreased (Fig. 4A-D). After 3 days of rehydration, the contents of total metabolites, organic acids, and other metabolites recovered to normal level (Fig. 4 A, C, and D). The content of amino acids also showed a significant increase after rehydration, but did not return to normal level (Fig. 4B).

Drought stress significantly decreased contents of aspartic acid, glycine, alanine, oxoproline, serine, and γ-aminobutyric acid. After rewatering, the contents of glycine, alanine, and γ-aminobutyric acid (GABA) increased significantly, but rehydration did not affect contents of aspartic acid, oxoproline, and serine (Fig. 5A). There were no significant differences in contents of threonine and valine between drought stress and rewatering (Fig. 5B). The content of proline decreased significantly after dehydration, but recovered to a significantly level after 3 days of rehydration (Fig. 5B). Dehydration and rehydration did not affect the content of hydroxyproline (Fig. 5C). Glutamine content significantly declined under drought stress and then further declined after 3 days of rehydration (Fig. 5C).

Drought stress significantly reduced the accumulation of aminomalonic acid, hydroxybutanoic acid, glyceric acid, vanillic acid, or butenedioic acid, but did not affect the accumulation of butanoic acid, propanedioic acid, lactic acid, malic acid, or butanedioic acid (Fig. 6A and B). Contents of glyceric acid and vanillic acid recovered to normal level and the contents of hydroxybutanoic acid and lactic acid exceeded normal levels after 3 days of rewatering (Fig. 6A and B). Drought and rewatering induced significant accumulation of citric acid (Fig. 6B). Contents of arabinose, ethanamine, ethanolamine, and rhamnose decreased significantly under drought stress, but could return to normal level after 3 days of rewatering (Fig. 7A and B). Contents of ribonic acid, arabinitol, and erythritol were not significantly affected by drought stress, but increased significantly after rewatering. As compared to well-watered condition, the content of ribitol or campesterol was maintained at a significantly higher or lower level under drought and re-watered conditions (Fig. 7). Figure 8 showed physiological changes and key metabolites in leaves of *Selaginella tamariscina* in response to dehydration and rehydration.

Discussion

In recent years, irrigation water resource is decreasing due to global warming and frequent extreme weather events worldwide, especially in many drought-prone regions. Drought stress not only affects plant morphology and yield, but also disturbs their physiological and metabolic processes. Better recoverability of plants from drought damage is beneficial for their adaptation to complex and changing

environments (Challabathula and Bartels 2013; Fang and Xiong 2015). As a typical extreme drought-tolerant plant, *S. tamariscina* provide important references for the understanding of underlying mechanisms of drought tolerance and resurrection. Rsults from the present study showed that a prolonged period of drought stress resulted in extreme declines in RWC, Chl content, Fv/Fm, and PI_{ABS} in leaves of *S. tamariscina* with a curled, dry, and yellowed phenotype. However, *S. tamariscina* plants could rapidly recover within 3 days of rehydration, as demonstrated by green and full unfolded leaves as well as recuperative physiological parameters. These findings were consistent with those of previous studies which demonstrated that *S. tamariscina* could quickly recover from dehydration to regain higher RWC, Chl content or photochemical efficiency after a short-term rehydration, indicating that *S. tamariscina* possesses a strong ability for post-drought recovery (Wang et al. 2010; Agduma and Sese 2016; Xu et al. 2018).

Plants produce a large amount of ROS which causes membrane lipid peroxidation and metabolic disorders under stressful conditions, even leading to plant death (Sharma et al. 2012). SOD, CAT, and POD are basal stress-defensive antioxidant enzymes for detoxifying ROS damage to plants. SOD is known as the first line of defense in antioxidant system, because it catalyzes the dismutation reaction of superoxide anions to produce H_2O_2 and O_2. CAT and POD serve as important scavengers of H_2O_2 in plants (Hasanuzzaman et al. 2020). Integrative genomic and transcriptomic analyses revealed that ROS generation and scavenging were the main pathways for acquisition of desiccation tolerance in *S. tamariscina* (Xu et al. 2018). Current findings showed that activities of SOD, CAT, and POD were significantly increased under drought stress, which was accompanied by a significant increase in MDA content as a product of membrane lipid peroxidation because of ROS-induced oxidative damage. Interestingly, the activities of SOD and CAT did not significantly decreased but remained at high levels after rewatering, and the activity of POD even further increased in leaves of *S. tamariscina* after 3 days of rehydration. For other resurrection plants, activities of SOD and CAT were maintained at significantly higher levels in *S. bryopteris* during dehydration, but returned to normal levels after rehydration (Pandey et al. 2010), which was consistent with the study of Shivaraj et al. about detached leaves of *S. brachystachya* in response to dehydration and rehydration (Neeragunda Shivaraj et al. 2021). These findings indicated that antioxidant enzymes were activated to alleviate dehydration-induced oxidative damage to *S. tamariscina*, and antioxidant enzyme system also played a protective role in repair mechanism during the process of *S. tamariscina* resurrection. Different resurrection plant species may exhibit differential resurrection mechanism of antioxidant defense.

In response to water deficit, plants accumulate various organic and inorganic osmoregulatory substances to maintain water potential (Ashraf et al. 2011; Chen and Jiang 2010). Most of organic osmolytes also play a positive role in osmoprotection such as various amino acids, sugars, and sugar alcohols, which stabilize intracellular environments (Li et al. 2017a). More than 30 organic metabolites were significantly changed when *S. tamariscina* was subjected to drought and recovered after rewatering. Although contents of amino acids, organic acids, and other metabolites significantly declined in leaves of *S. tamariscina* under drought stress, the contents of these metabolites basically returned to

normal levels after rehydration, indicating their potential roles in resurrection of *S. tamariscina*. It's worth noting that citric acid and ribitol significantly accumulated in response to dehydration and still kept at higher levels after rehydration. Citric acid is an intermediate in tricarboxylic acid cycle, and the maintenance of citric acid content was propitious to energy metabolism for defensive response in non-resurrection plant species under water deficit condition (Li et al. 2017b; Li et al. 2019; Merewitz et al. 2011). In resurrection plant *S. lepidophylla*, citric acid as well as sugar alcohols including ribitol and erythritol increased in leaves during rehydration, which could be a critical mechanism of resurrection due to positive roles of these metabolites in the maintenance of energy metabolism, OA, and osmoprotection (Yobi et al. 2013). In *S. tamariscina*, the accumulations of citric acid and ribitol could protect cells against dehydration and also exhibited beneficial function for recovery.

Many amino acids, sugars, and other metabolites decreased sharply in leaves of *S. tamariscina* under drought stress, but their contents recovered quickly and even exceeded normal levels after rehydration, as shown in Fig. 8. It has been reported that enhanced carbohydrate metabolism could be a positive strategy for adaptation to desiccation through comparing differential genes in drought-tolerant *S. tamariscina* and drought-sensitive *S. moellendorffii* (Gu et al. 2019). Desiccation-tolerant *S. lepidophylla* could also accumulate more sugars and sugar alcohols than desiccation-sensitive *S. moellendorffii* when leaf RWC declined from 100–50% (Yobi et al. 2012). In addition, multiple amino acids including alanine, glycine, GABA, and proline significantly increased in non-resurrection *Brasska napus* under drought stress and then reduced after rewatering (Good and Zaplachinski 1994). However, severe desiccation resulted in declines in glycine, valine, and isoleucine in resurrection plant *Haberlea rhodopensis*, but their levels could quickly recover after rehydration (Ivanov et al. 2014). Desiccation induced the accumulation of proline in leaves of *S. bryopteris*, and its content was still kept at a higher level than hydrated control after rehydration (Pandey et al. 2010). In our current study, the *S. tamariscina* could revive from an extreme low cellular water status associated with accumulation of amino acids, sugars, and other organic metabolites such as glycine, alanine, GABA, proline, vanillic acid, and arabinose after rehydration. Although it has been widely reported about beneficial functions of proline and GABA on regulating drought tolerance as critical osmolytes, antioxidants, and stress signaling molecules to induce defensive responses in non-resurrection plants under water deficit (Jiang et al. 2023; Jiang 2023; Hasan et al. 2021), positive roles of these key metabolites in rehydration in resurrection plant species deserve to be investigated in the future.

Conclusions

Severe drought stress resulted in extreme declines in RWC, Chl content, Fv/Fm, and PI_{ABS} in leaves of *S. tamariscina* which could rapidly recover within 3 days of rehydration. SOD, POD, and CAT were activated by dehydration and also maintained at significantly higher levels in response to rehydration, thereby alleviating oxidative damage to *S. tamariscina* plants. The results indicated that enhanced antioxidant defense system could be one of the main pathways for acquisition of desiccation tolerance and resurrection capacity. In addition, drought-induced accumulations of citric acid and ribitol could protect cells against dehydration and also improve energy metabolism during dehydration and rehydration.

Although most of organic metabolites decreased sharply in leaves under drought stress, but *S. tamariscia* has the ability to quickly regain metabolic competency after rehydration, which could be the important mechanism of resurrection due to positive roles of these metabolites in OA, osmoprotection, antioxidant, and energy metabolism.

Declarations

Authors contribution

ZL structured the experimental concept, provided reagents, consumable materials and technological guidance, and revised the manuscript. YX and YC conducted the experiments and analyzed the data. YX wrote the first draft of the manuscript.

Funding

This research was supported by National Undergraduate Training Program for Innovation and Entrepreneurship (202210626027).

All other authors declare no competing interests.

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Figures

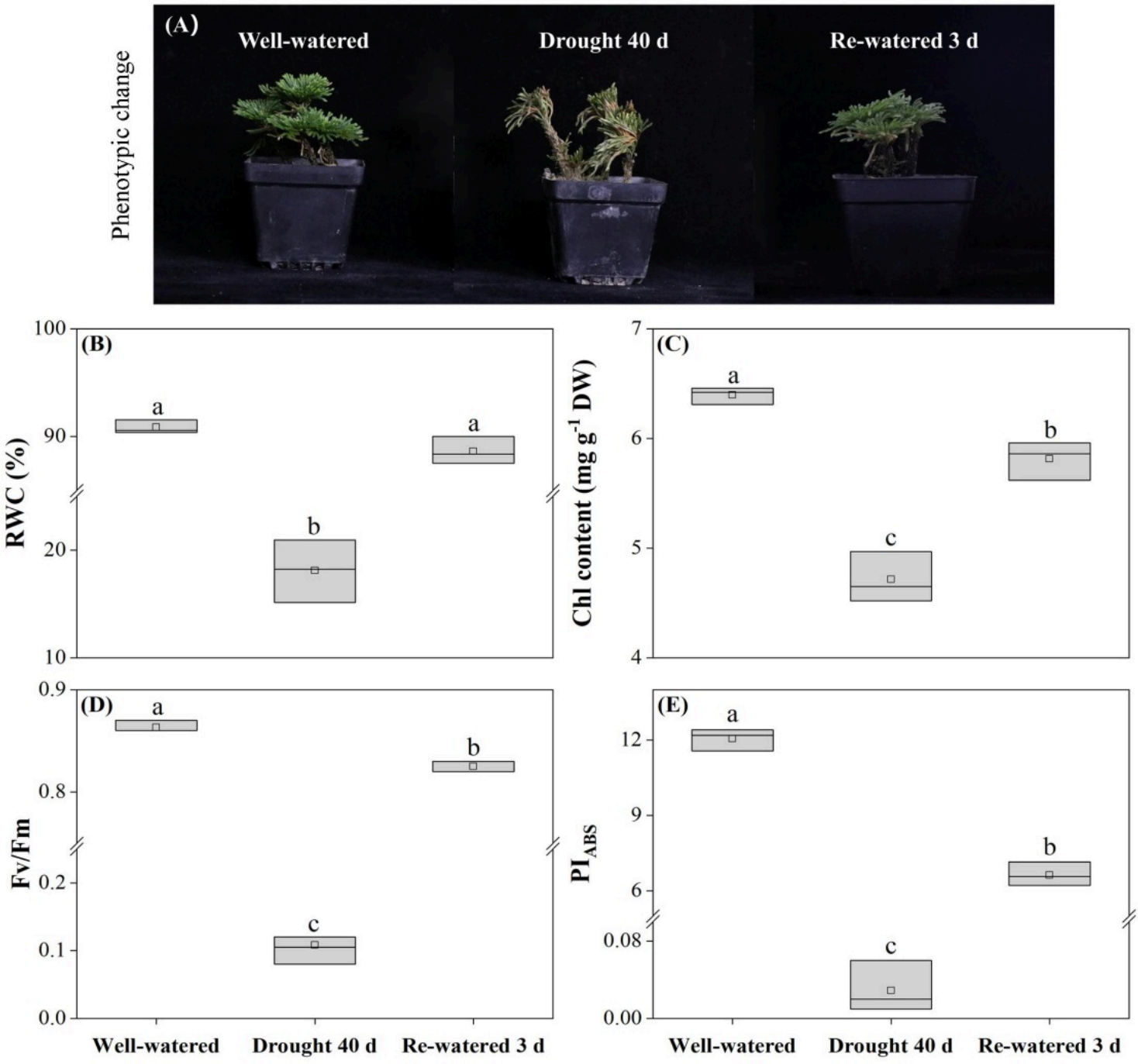

Figure 1

Changes in plant morphology (A), leaf RWC (B), Chl content (C), Fv/Fm (D), and PI_{ABS} (E) in leaves of *Selaginella tamariscina* under normal condition, dehydration, and rehydration.

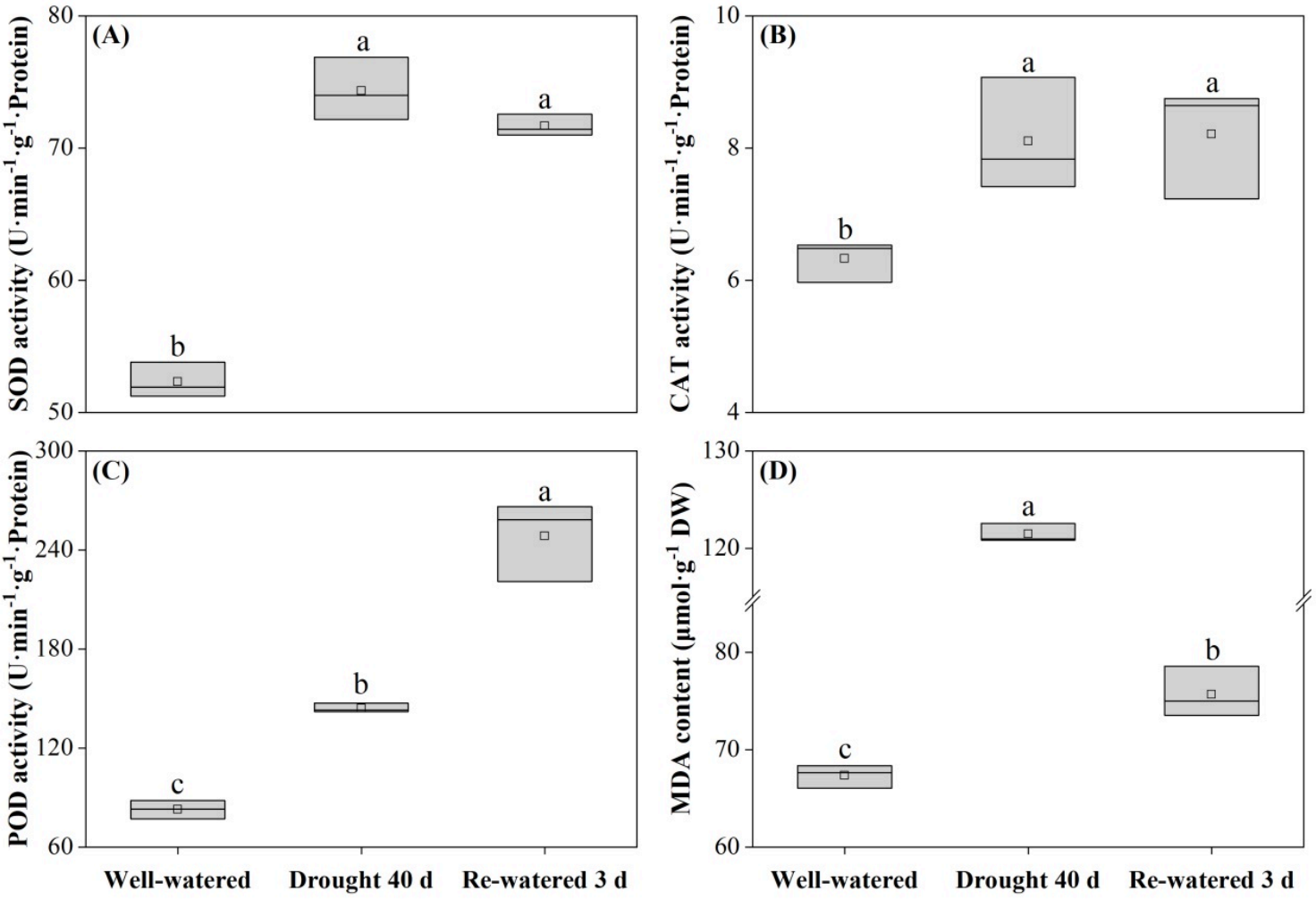

Figure 2

Changes in SOD activity (A), CAT activity (B), POD activity (C), and MDA content (D) in leaves of *Selaginella tamariscina* under normal condition, dehydration, and rehydration.

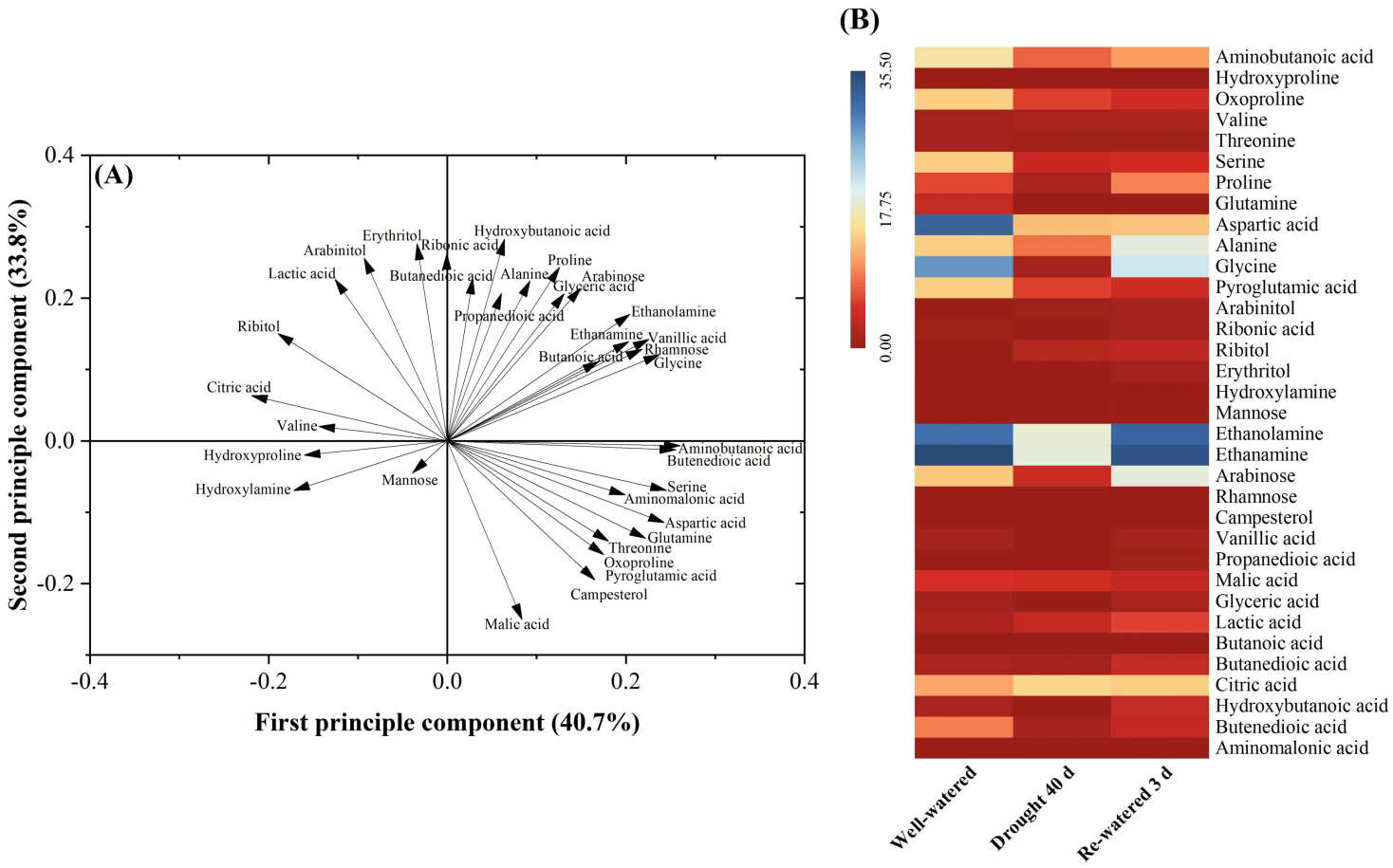

Figure 3

Principal component analysis and heat map of 33 metabolites in in leaves of *Selaginella tamariscina* under normal condition, dehydration, and rehydration.

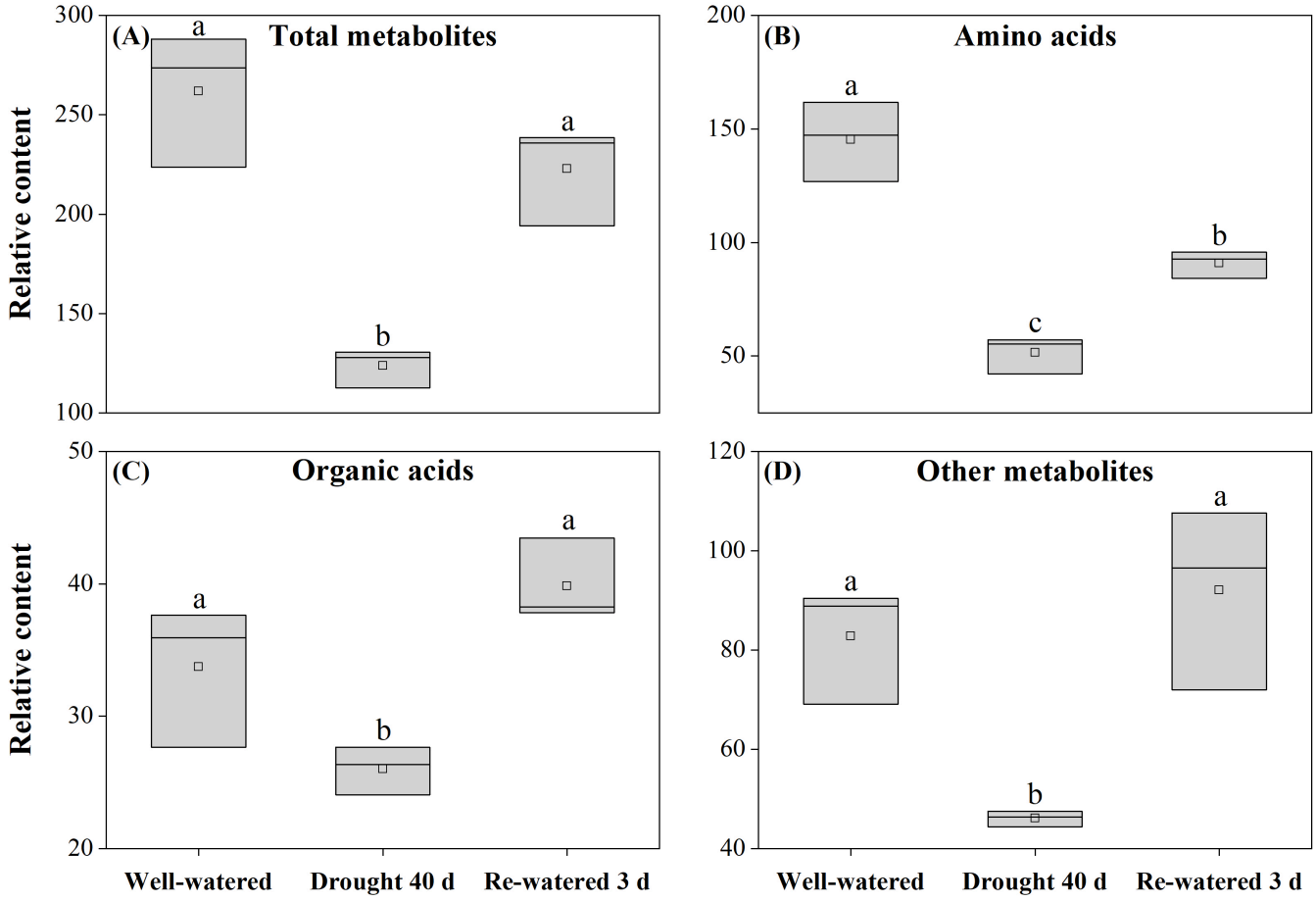

Figure 4

Changes in total metabolite content (A), amino acids content (B), organic acids content (C) and other metabolites content (D) in leaves of *Selaginella tamariscina* under normal condition, dehydration, and rehydration.

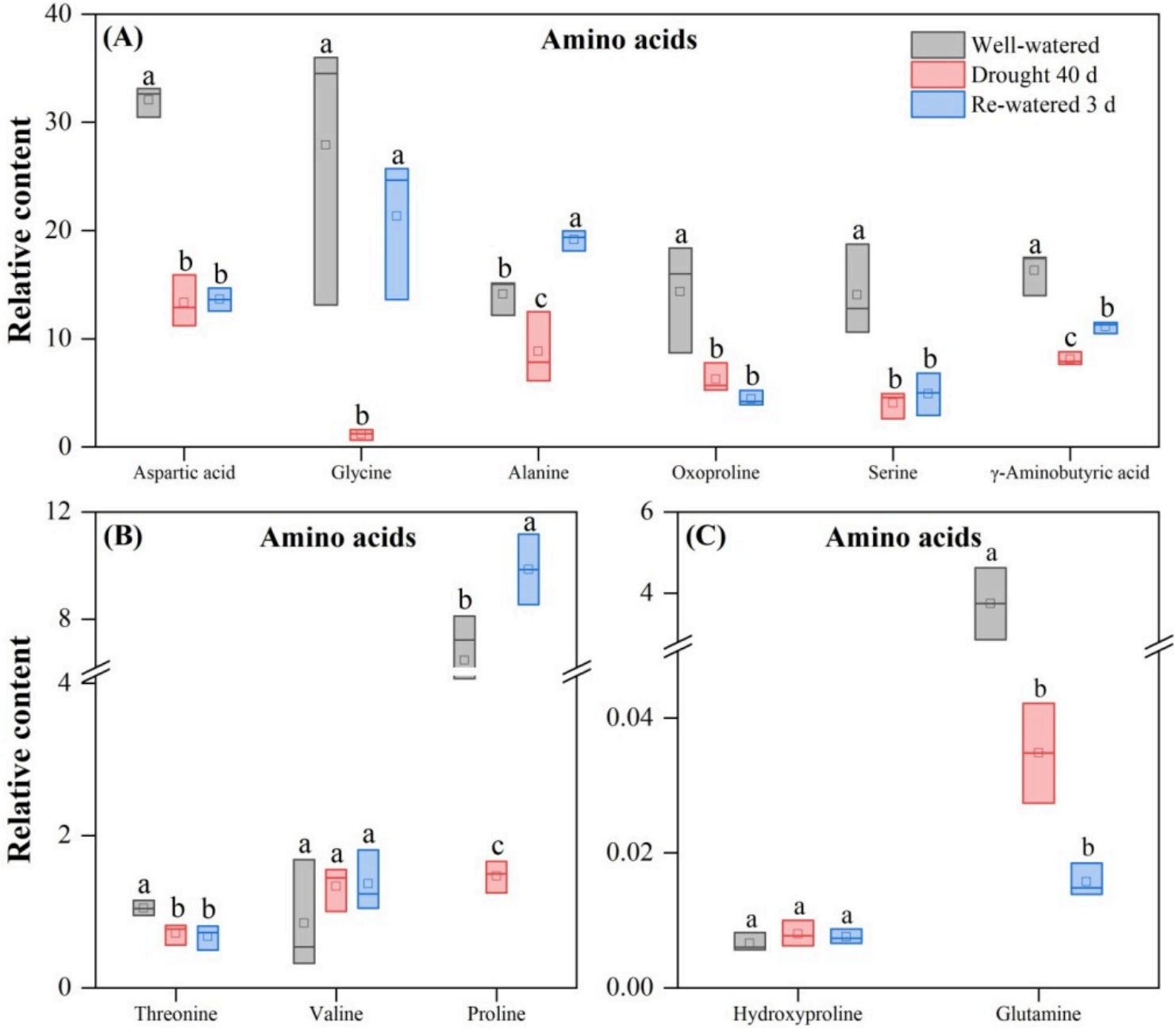

Figure 5

Changes in relative contents of amino acids in leaves of *Selaginella tamariscina* under normal condition, dehydration, and rehydration.

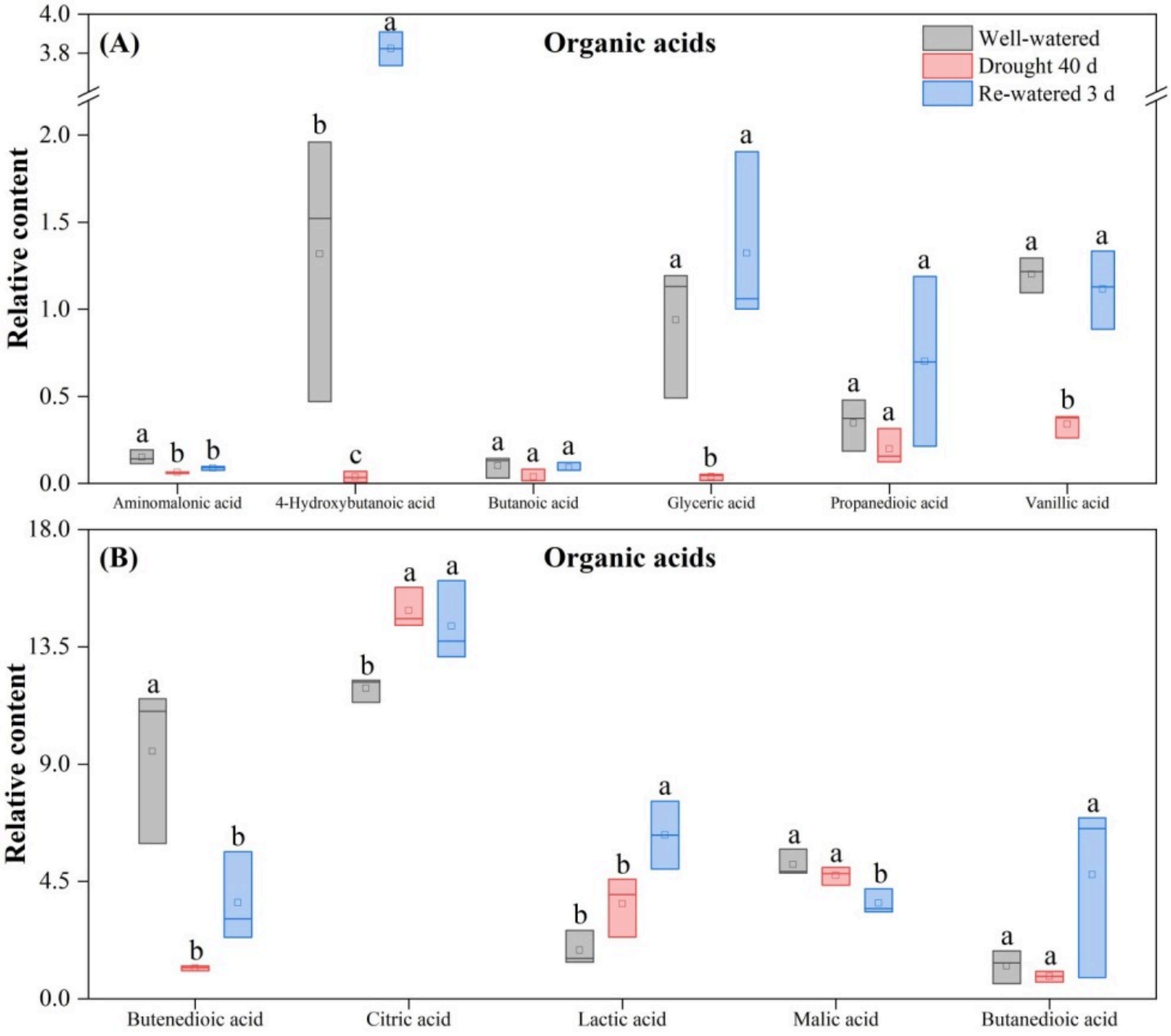

Figure 6

Changes in relative contents of organic acids in leaves of *Selaginella tamariscina* under normal condition, dehydration, and rehydration.

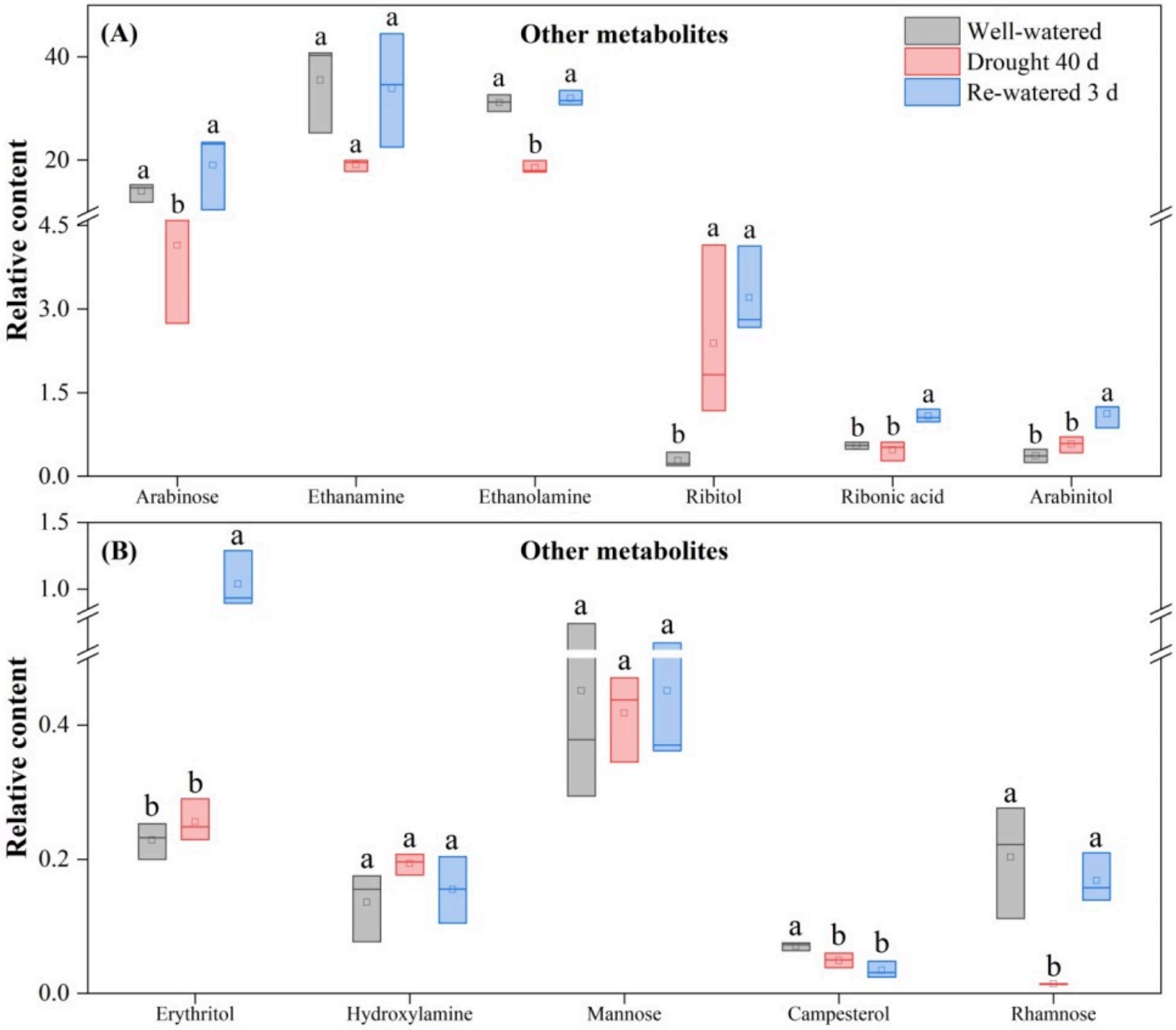

Figure 7

Changes in relative contents of other metabolites in leaves of *Selaginella tamariscina* under normal condition, dehydration, and rehydration.

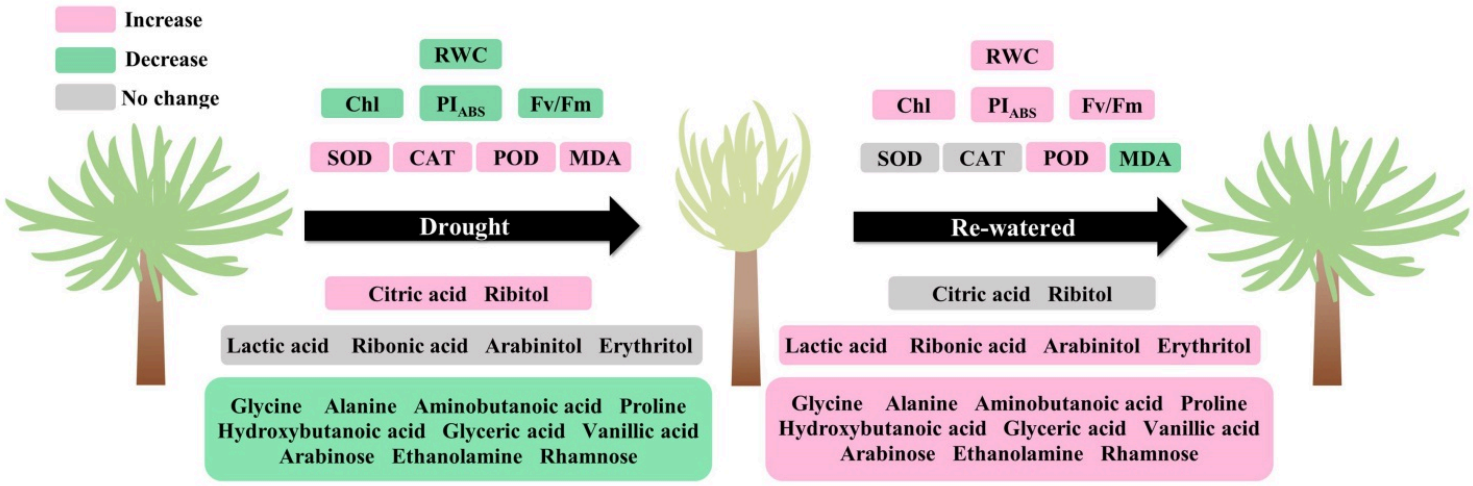

Figure 8

Physiological changes and key metabolites in leaves of *Selaginella tamariscina* in response to dehydration and rehydration.

Supplementary Files

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- 2Supplementarytable.docx