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Salinity stress increases secondary metabolites and enzyme activity in safflower

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ABSTRACT

Safflower (*Carthamus tinctorius* L.) is a salt-tolerant crop cultivated worldwide. However, little information is available on their tolerance mechanism. Owing to decreasing arable land, cultivation of safflower in saline environments might be needed in order to promote crop biomass and secondary metabolites production. The physiological response of the selected species should be examined in order to improve environmental options, especially facing salinity stress. A hydroponic culture experiment was designed to investigate the changes in fundamental physiological processes and secondary metabolite accumulation induced by salinity in safflower (Cv. Space-1). Safflower seedlings were grown in half-strength Hoagland solution and exposed to 0, 50, 100 and 150 mM NaCl for 30 days. NaCl concentrations <100 mM did not affect the growth of safflower seedlings in terms of plant height, root length and plant dry weight, as well as the relative growth rate; however, it did increase the medicinal flavonoid content in leaves. Although the Na^+ concentration was considerably increased under salt stress, higher K^+ and Ca^{2+} concentrations in the leaves and shoots were observed simultaneously. The K^+/Na^+ ratio was significantly decreased with increasing salinity, whereas NaCl treatments all had K^+/Na^+ values >0.5, suggesting ion homeostasis. Chl-*a* and Chl-*b* contents in safflower leaves were negatively affected by salinity, whereas the carotenoid contents remained unchanged with NaCl concentrations <100 mM. Additionally, SOD, CAT, POD and T-AOC activities in safflower leaves were markedly increased for all of the NaCl treatments. The soluble sugar and protein contents in safflower leaves under salt stress were much higher than the control and increased significantly with increasing salinity. The present study demonstrates that *C. tinctorius* var. Space-1 withstands moderate doses of NaCl in the medium and that sound physiological responses of the plant help to increase secondary metabolite accumulation under salt- stressed conditions.

1. Introduction

Soil salinity is a major abiotic stress that severely affects crop production worldwide (Kumar et al., 2007). A considerable amount of land in the world is affected by salinity and more than 45 million hectares (M ha) of irrigated land which account to 20% of total land have been damaged by salt and 1.5 M ha are taken out of production each year due to high salinity levels in the soil (Munns and Tester, 2008). For developing countries, the highly efficient use of saline agriculture is a major scientific problem for local governments and agronomists. Nowadays, China has considered using coastal saline lands to develop salt-tolerant plants for bioenergy and medicine (Zhao et al., 2010; Liu et al., 2010). Safflower (*Carthamus tinctorius* L.) is an annual medicinal and aromatic oilseed crop (Kumar and Kumari, 2005). Its flowers have many medicinal properties for several chronic diseases and they are widely used in Chinese herbal preparations (Li and Mundel, 1996). Although safflower is cultivated in an area of approximately 55,000 ha per year (Wang and Du, 2001), its physiological adaption to salt stress have not yet been fully elucidated.

High salt concentrations cause various events that negatively impact agricultural production, such as delays in plant growth and development, inhibition of enzymatic activities and a reduction in the photosynthetic rate (Lee et al., 2013). It has been reported that excess Na^+ and Cl^- in the soil solution caused osmotic stress, and plants accumulate osmotically active compounds to lower the osmotic potential (Ahmad and Sharma, 2008). Salt induced osmotic stress is responsible for the oxidative stress caused by

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reactive oxygen species (ROS). The harmful effect of ROS is the oxidative attack on proteins, resulting in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products and increased susceptibility to proteolysis (Ahmad et al., 2010). To mitigate ROS-mediated oxidative damage, plants have developed a complex antioxidant defence system, including low-molecular mass antioxidants, such as ascorbate, reduced glutathione, tocopherol, carotenoids, and flavonoids, as well as antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), and catalase (CAT) (Núñez et al., 2003; Azevedo-Neto et al., 2006).

In this study, the changes in plant growth, photosynthesis, antioxidant enzymes, osmosis and metabolite were evaluated. These data resulted in comprehensive physiological and metabolic insights into the response induced by salt stress in safflower plants. The results of this particular study may provide valuable information regarding the mechanism by which *C. tinctorius* L. var. Space-1 adapts to NaCl stresses.

2. Materials and methods

2.1. Plant material, growth conditions and harvesting

Safflower (*C. tinctorius* var. Space-1) seeds were collected from Bozhou herbal medicine market in September 2012. They were preserved naturally in the Marine Environment and Ecology Lab (NAU) until the experiment started on March 9, 2013. Surface-sterilised with 2.5% sodium hypochlorite solution and rinsed with distilled water, the seeds were then transferred to sterile moist filter paper after swelling in distilled water at 25 °C for 12 h. The seeds were put in a glass petri dish for germination at 28 °C for 12 h in the daylight and 22 °C for 12 h in the dark. 72 h later, the seeds were germinated, and then consistently germinated seeds selected and cultivated in holes within Styrofoam boards filled with organic media comprising humus, perlite and vermiculite at a ratio of 4:1:1.

The growth of safflower seedlings was under the following conditions: 28 °C/22 °C day and night temperatures, respectively, and a relative humidity of 70–80% in a semi-controlled plant greenhouse in the Pailou Base(NAU). When the new 4th leaf emerged, 16 seedlings with consistent appearance were chosen and transferred to 1/2 Hoagland nutrient solution for further training. The nutrient solution was aerated continuously and replaced every two days. For the salt treatments, soluble NaCl was gradually included in the culture medium until the salt concentrations reached 50, 100 and 150 mM. The hydroponic experiment was arranged as a factorial setup based on a completely randomised design with four replications. The safflower seedlings were exposed to salt stress by adding NaCl to the culture medium when the plants were 2-weeks old on March 23, and the plants were harvested on April 22.

2.2. Growth analysis

In this experiment, four individual plants for each treatment were sampled for the plant growth analysis. After harvest at 30 d, the fresh weight (FW) and dry weight (DW), which was measured after the samples were dried at 70 °C for 72 h, were measured to calculate the relative growth rate (RGR) of the seedlings according to the formula given by Hunt et al. (2002). Four leaf disks were obtained from the *C. tinctorius* plants and their fresh weight (FW) were determined. The leaf discs were floated on de-ionised water for 6 h under low irradiance and the turgid tissue was then quickly blotted dry prior to determining the turgid weight (TW). The dry weight (DW) was determined after oven drying at 70 °C for 72 h, the time point at which a constant weight was reached. The relative water content (RWC) was calculated using the following formula: $RWC(\%) = (FW - DW) \times 100/(TW - DW)$ (Smart and Bingham, 1974).

2.3. Ion concentration analysis

Plant tissue samples were dried at 65 °C for 6 d, weighed, and ground in a Wiley mill to pass through a 40 mesh screens. Subsamples were digested using a hydrogen peroxide (H_2O_2)/nitric acid (HNO_3) digests according to Huang and Schulte (1985) and analysed by inductively coupled emission spectroscopy at the Nanjing Agricultural University life science Analysis Laboratory (ICP-OES, Iris plasma spectrophotometer).

2.4. Measurement of photosynthetic pigments

Safflower leaf tissues were ground with liquid nitrogen, and then 1 ml 100% acetone was added for chlorophyll extraction. After vigorous vortexing at 4 °C for 1 h in the dark, the cell debris was removed by centrifugation at 15,000 × *g* at 4 °C for 15 min. The chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total chlorophyll (T-Chl) concentrations were calculated using the equations of Lichtenthaler as follows: Chl $a = 11.24 \times A661.6 - 2.04 \times A644.8$; Chl $b = 21.13 \times A644.8 - 4.19 \times A661.6$; and $tChl = 18.09 \times A644.8 + 7.05 \times A661.6$ (Lichtenthaler, 1987). Total carotenoids were analysed as described by Mencarelli and Saltveit (1988).

2.5. Antioxidant enzyme assays

To determine the antioxidant enzyme activities, 0.5 g safflower leaves were homogenised with ice cold 50 mM KPi (pH 7.0) containing 0.1 mM EDTA, 1% (w/v) polyvinyl-pyrrolidone (PVP) and 0.5% (v/v) Triton X-100 at 4 °C. The homogenate was filtered through four layers of cheesecloth and centrifuged at 14,000 × *g* for 15 min at 4 °C. The supernatant was collected for the determination of antioxidant enzyme activities, and stored at −80 °C until further analyses (Bradford, 1976). The CAT activity was assayed by measuring the initial H_2O_2-scavenging rate. The extinction coefficient for H_2O_2 at 240 nm is 40 mM^{-1} cm^{-1} (Bergmeyer, 1970). The SOD activity was assayed according to the method using pyrogallol as a substrate (Kwak et al., 1995). The molar extinction coefficient of purpurogallin is 2.47 mM^{-1} cm^{-1}. POD activity was done according to the method of Plewa et al. (1991) based on the amount of tetraguaiacol absorbed after formation by oxidation of guaiacol catalysed by this enzyme in 3 min at a wavelength of 470 nm using an extinction coefficient of tetraguaiacol. Total antioxidative capability (T-AOC) was measured by the method of ferric reducing-antioxidant power assay (Benzie and Strain, 1996) and detected at 520 nm with the spectrophotometer.

2.6. Measurement of soluble sugar and protein contents

To measure the soluble sugar content, 0.5 g dry leaves was homogenised with 5 ml 95% ethanol. One-tenth millilitre alcoholic extract stored in the refrigerator was mixed with 3 ml anthrone (150 mg anthrone, 100 ml 72% sulphuric acid, W/W). The samples were the placed in a boiling water bath for 10 min. The light absorption of the samples was estimated at 625 nm using a spectrophotometer. The soluble sugar contents were determined using a glucose standard and are expressed as mg g^{-1} DW of leaves. From each sample, 250 mg were extracted in 0.8 ml Tris-boric buffer (0.09 M Tris, 0.08 M boric acid, and 0.93 g l^{-1} Na_2EDTA) and 0.8 ml 40% w/v sucrose. The extraction was then centrifuged at 10,000 × *g* for 10 min. The supernatant was mixed with an equal volume of Laemmli solution (1 M Tris (pH = 8.8), 0.4 g SDS, 0.8 g glycerol, and 0.9 ml 2-ME (mercaptoethanol) in 10 ml ddH_2O), heated in boiling water for 5 min, and frozen until further use. The total soluble