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Intrinsic water use efficiency controls the adaptation to high salinity in a semi-arid adapted plant, henna (*Lawsonia inermis* L.)

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ABSTRACT

Adaptation to salinity of a semi-arid inhabitant plant, henna, is studied. The salt tolerance mechanisms are evaluated in the belief that gas exchange (water vapor and CO_2) should play a key role on its adaptation to salt stress because of the strong evaporation conditions and soil water deficit in its natural area of distribution. We grow henna plants hydroponically under controlled climate conditions and expose them to control (0 mM NaCl), and two levels of salinity; medium (75 mM NaCl) and high (150 mM NaCl). Relative growth rate (RGR), biomass production, whole plant and leaf structure and ultrastructure adaptation, gas exchange, chlorophyll fluorescence, nutrients location in leaf tissue and its balance in the plant are studied. RGR and total biomass decreased as NaCl concentration increased in the nutrient solution. At 75 mM NaCl root biomass was not affected by salinity and RGR reached similar values to control plants at the end of the experiment. At this salinity level henna plant responded to salinity decreasing shoot to root ratio, increasing leaf specific mass (LSM) and intrinsic water use efficiency (iWUE), and accumulating high concentrations of Na^+ and Cl^- in leaves and root. At 150 mM NaCl growth was severely reduced but plants reached the reproductive phase. At this salinity level, no further decrease in shoot to root ratio or increase in LSM was observed, but plants increased iWUE, maintaining water status and leaf and root Na^+ and Cl^- concentrations were lower than expected. Moreover, plants at 150 mM NaCl reallocated carbon to the root at the expense of the shoot. The effective PSII quantum yield [Y(II)] and the quantum yield of non-regulated energy dissipation [Y(NO)] were recovered over time of exposure to salinity. Overall, iWUE seems to be determinant in the adaptation of henna plant to high salinity level, when morphological adaptation fails.

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Introduction

Salt-affected soils are present in the majority of cultivated lands of the world as a result of the continuous use of fertilizers (FAO, 2008). It is especially relevant in the Mediterranean area, where water resources are increasingly rare, and saline water mixed with fertilizers is used in agriculture. The accumulation of dissolved salts in the soil water inhibits plant growth (Tester and Davenport, 2003). Sodium (Na^+) and chloride (Cl^-) are the two key ions responsible for osmotic, ion-specific damage and nutritional disorders (Munns and Tester, 2008) that trigger the growth rate reduction. Plants can display a plethora of mechanisms in response to salinity. Cheeseman (1988) designated the mechanism of interest as (a) those involved in transport and in the control and integration of Na^+ acquisition and allocation in plants and (b) those involved in readjustment of other aspects of metabolism, especially carbon. These mechanisms will provide the plant with the ability to exclude salt from the shoot (Na^+ excluders), tolerate high concentrations of salt in the leaf (Na^+ includers) or tolerate osmotic stress (Rajendran et al., 2009).

Henna plant is a flowering shrub of the *Lytheraceae* family, which is distributed in dry tropical and subtropical zones in Africa, Asia and the Middle East (Anon, 1986). Farmers in hot arid regions find henna a reliable cash crop when drought and heat withers other crops. This plant belongs to continental oases where water shortage and saline soils constitute the essential limiting

Abbreviations: A, net photosynthetic rate; Chl, chloroplast; Ct, cytoplasm; DAS, days after sowing; DW, dry weight; E, epidermis; FW, fresh weight; gs, stomatal conductance; LB, lipid body; LD, leaf density; LSM, leaf specific mass; M, mitochondria; PP, palisade parenchyma; PPFD, photosynthetic photon flux density; RGR, relative growth rate; RWC, relative water content; S, starch grain; SP, spongy parenchyma; TEM, transmission electron microscopy; TW, turgid weight; V, vacuole; iWUE, intrinsic water use efficiency; Y(II), quantum yield of photochemical energy conversion in PS II; Y(NO), quantum yield of non-regulated non-photochemical energy loss in PS II; Y(NPQ), quantum yield of regulated non-photochemical energy loss in PS II.

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factors of agricultural production (Askri et al., 2010). The studies addressing the physiological and morphological adaptations to salinity of non-halophyte semi-arid adapted plants show that, in general, these plants adapt to salinity by decreasing shoot dry weight, leaf area, and maintaining turgor via osmotic adjustment and stomatal closure (Alarcon et al., 2006; Alvarez et al., 2012; Navarro et al., 2007). Because henna plants survive strong evapotranspiration conditions, we hypothesized that the tolerance mechanisms developed in salt stress conditions should mainly involve stomata conductance regulation and CO_2 assimilation. Here we provide a fundamental biological understanding of its mechanisms of response to salt stress and establish the salt-tolerance of henna plants. To the best of our knowledge no scientific literature on salt stress response of henna plant has existed until now, and this semi-arid adapted plant could be useful to test the relative importance of the mechanisms of adaptation to drought stress in the adaptation to salt stress. We think that the information derived from this study could also be helpful for gardening and erosion control projects in lands elevated sodium and chloride concentration in the soil.

Materials and methods

Plant material and growth conditions

Seeds of henna plants (*Lawsonia inermis* L.) were collected from Gabes (Tunisia) and germinated at room temperature for two weeks on moist filter paper in Petri dishes wrapped with parafilm®. Then seedlings were growth in controlled environmental chamber in 5 L containers with aerated modified nutrients solutions (Epstein and Bloom, 2005); macronutrients (mM): 1.25 $CaSO_4$, 0.2 KNO_3, 0.2 NH_4Cl, 0.75 $MgSO_4$, 0.25 KH_2PO_4, 0.75 K_2HPO_4; and micronutrients (μM): 50 KCl, 25 H_3BO_3, 2 $MnSO_4{\cdot}H_2O$, 2 $ZnSO_4{\cdot}7H_2O$, 0.5 $CuSO_4{\cdot}5H_2O$, 0.5 Na_2MoO_4, and 0.2 g L^{-1} Fe-EDDHA (Ferro-codifit, Agrosafor, Spain). After one week, plants were moved to 28 L containers, placing 8 plants per container. The nutrient solution in the 28 L containers was replenished every 3–4 days and the pH of the nutrient solutions was adjusted every two days to 5.8. Plants grew in a controlled growth chambers, with a day temperature of 23 °C and 16 °C at night, and relative humidity of 60% at day and 80% at night and a photoperiod of 16 h with a photosynthesis flux density of 400 μmol m^{-2} s^{-1} at plant height.

Treatments and experimental design

Three treatments; 0, 75 and 150 mM NaCl were initiated in 25-day old plants, adding 25 mM NaCl twice a day until reaching 75 mM and 150 mM in the salinity treatments. The experimental design involved 3 treatments, each treatment had 3 containers, and each container had 8 plants.

Non-destructive plant growth analysis and biomass measurements

We calculated relative growth rate (RGR) from the change in the natural log of fresh mass of each plant over time. Shoot and root biomass were determined by harvesting 12 plants at 43 and 53 days after sowing (DAS). Subsamples of leaves (neither old nor young leaves) and root were dried in an air force oven.

Leaf specific mass and relative water content

Leaf disks samples (1.6 cm^2) were taken with a sharp cork borer at midday from 12 plants per treatments at 48 DAS. Top-most fully expanded leaves were sampled avoiding large veins. Samples were placed in a pre-weight microtube vial and quickly weighed (FW). Then the sample was immediately hydrated to full turgidity for 3–4 h by floating on de-ionized water in a closed Petri dish under normal room light and temperature. Afterwards, hydration samples were well dried with filter tissue paper and immediately weighed to obtain fully turgid weight (TW). Samples were oven dried for a minimum of 24 h at 70 °C to determine dry weight (DW). Leaf specific mass (LSM, the ratio of leaf mass to leaf area) was obtained from the fresh weight (FW) and DW and leaf dish area. Relative water content was calculated as $RWC\ (\%) = [(FW - DW)/(TW - DW)] \times 100$.

Gas exchange and chlorophyll fluorescence

The mid-portion of the second or third fully expanded leaf was placed into the PLC6 head-cuvette of the CIRAS-2 portable photosynthesis system (PP SYSTEMs, AMESBURY; MA, USA). Simultaneous measurements of net CO_2 assimilation rate (A) and H_2O exchange rate (gs) were made at 400 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at 42 and 52 DAS. Intrinsic water use efficiency (iWUE) was calculated as the ratio of photosynthetic assimilation rate (A) to stomatal conductance for water vapor (gs) (Ehleringer et al., 1993), which quantifies the amount of carbon assimilated per unit leaf area per unit time for a per unit cost of water. Leaf temperature was maintained at 23 °C. On the single leaf used for gas exchange, dark-adapted maximum fluorescence (Fm) and minimum fluorescence (Fo) and light-adapted, steady-state chlorophyll fluorescence (F) and maximum (Fm′) were made with the MAXI version of the IMAGING-PAM M-Series (Walz, Effeltrich, Germany). The maximum quantum efficiency of PSII was calculated as $Fv/Fm = (Fm - Fo)/Fm$. The quantum yield of photochemical energy conversion in PS II [Y(II)] was calculated from (Genty et al., 1989) and the quantum yield of regulated [Y(NPQ)] and non-regulated [Y(NO)] non-photochemical energy loss in PS II were calculated according to Kramer et al. (2004). The ratio Y(NPQ)/Y(NO), a measure of the capacity of photoprotective reactions (klughammer et al., 2008) was also calculated. Fo′ was estimated using the approximation of Oxborough and Baker (1997), $Fo' = Fo/(Fv/Fm + Fo/Fm')$, where Fv is the variable fluorescence ($Fv = Fm - Fo$).

Transmission electron microscopy (TEM)

In the same leaves used for LSM and RWC sections of leaves (1 mm × 1 mm from the most recent fully expanded leaves) were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 2.5 h. After three washes with the buffer, the samples were post-fixed in 1% osmium tetroxide, in the same buffer, for 2 h. After this, three washes with phosphate buffer were performed. All fixed tissues were dehydrated in a graded series of ethanol, then infiltrated, first with a propylene oxide and then with propylene oxide and Spurr's resin mixture. The samples were then immersed in Spurr's resin overnight at 4 °C. Finally, the samples were embedded in Spurr resin. Blocks were sectioned on a Leica EM UC6 ultramicrotome, collected on copper grids and stained with uranyl acetate followed by lead citrate. Sections were examined using a Philips Tecnai 12 transmission electron microscope.

White light microscopy

Semi-thin sections (1 μm thick) of material prepared for TEM (see earlier) were cut with a Leica EM UC6 ultramicrotome. The sections were stained with toluidine blue: 2–5 min in 1% (w/v) toluidine blue, in 1% borax solution, at 60 °C. The sections were rinsed with water and air in a dust-free environment, before being mounted in DPX and observed with a Leica DMR light microscope.

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