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Canopy position determines the photoprotective demand and antioxidant protection of leaves in salt-stressed *Salvia officinalis* L. plants

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ABSTRACT

The effects of salt stress and leaf canopy position on mechanisms of photo- and antioxidant protection, including changes in photosynthetic pigments and low-molecular-weight chloroplastic antioxidants, were examined in sage (*Salvia officinalis* L.) plants exposed to two doses of NaCl (50 mM and 100 mM) for 5 weeks. Sage appeared to be a moderately salt-resistant glycophyte. The addition of 100 mM NaCl to the nutrient solution reduced total leaf biomass, the number of leaves, leaf water potential, net photosynthesis, stomatal conductance and chlorophyll levels. However, the malondialdehyde levels, which indicate the extent of lipid peroxidation, did not increase in plants treated with either 50 mM or 100 mM NaCl, relative to controls. In the plants treated with 100 mM NaCl, the accumulation of Na^+ in the leaves occurred in parallel with a drastic reduction in the net CO_2 assimilation rates, but also with the activation of mechanisms of photo- and antioxidant protection, including xanthophyll cycle de-epoxidation and the accumulation of tocopherols and phenolic diterpenes. Furthermore, we examined the extent to which canopy position determines the photoprotective demand and antioxidant protection of leaves, and how salinity affects this demand. The lower leaves showed a lower photoprotective demand than the upper leaves. However, the former showed higher lipid peroxidation than the latter under salt stress, which suggests that the lower, older leaves suffer from greater photo-oxidative stress than the upper, younger ones, despite being located in areas with a lower photoprotective demand within the canopy. We concluded that leaf position in the canopy should be carefully considered in studies aimed at unravelling mechanisms of salt-stress tolerance in plants.

1. Introduction

It is estimated that more than 6% of the world's land and 30% of the world's irrigated areas suffer from salinity, a problem that is expected to increase in the context of global change, particularly in the arid and semi-arid regions of the world (UNESCO, 2007; Pereira et al., 2009). Salinity reduces plant growth by decreasing shoot water potential and altering several metabolic activities at the cellular level, including the inhibition of enzymes, solute accumulation, specific ion effects or a combination of all these factors (Munns, 2002; Vinocur and Altman, 2005). The reduction in productivity observed for several plant species exposed to salinity is often associated with reduced photosynthetic capacity, due to both stomatal closure and biochemical alterations (Strasser et al., 1995; Belkhodja et al., 1999; Delfine et al., 1999; Loreto et al., 2003). In addition, these effects on photosynthesis increase the amount of excess excitation energy in chloroplasts (Mateo et al., 2004), which, if not safely dissipated, may result in PSII damage because of an over-reduction of reaction centres and an inhibition of the PSII repair process (Demmig-Adams and Adams, 1992; Strasser et al., 1995; Asada, 2006; Takahashi and Badger, 2011).

Protection against excess energy in plants depends on several mechanisms, such as the activation of xanthophyll cycle-dependent energy dissipation, activation of cyclic electron transport, the production and detoxification of singlet oxygen, activation of the Mehler reaction and the use of alternative sinks for ATP and NADPH such as photorespiration or the production of secondary metabolites (Demmig-Adams and Adams, 1996; Asada, 2006; Peñuelas and Munné-Bosch, 2005; Takahashi and Badger, 2011). However, the uncontrolled production of reactive oxygen species (ROS), such as singlet oxygen in PSII, can alter cellular metabolism through the oxidation of proteins, nucleic acids and lipids, thereby leading to the degradation of essential membrane components and disturbances in membrane permeability (Foyer

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et al., 1994; Osmond et al., 1997; Asada, 2006). To prevent ROS from damaging cellular components, plants have developed multiple detoxification mechanisms, including the production of various enzymes and the synthesis of antioxidant molecules. Indeed, chloroplasts contain low-molecular-weight lipophilic antioxidants, including carotenoids and tocopherols, which protect membranes from oxidative damage (Havaux, 1998; Asada, 1999; Trebst, 2003; Falk and Munné-Bosch, 2010). Carotenoids are known to protect cellular structures in plant response to a number of environmental stresses (Havaux, 1998; Wahid, 2007), not only by dissipating excess energy in chloroplasts as heat *via* the xanthophyll cycle-dependent mechanism, but also by preventing the chlorophyll-photosensitized formation of singlet oxygen (by intercepting chlorophyll triplet states) and scavenging any additional singlet oxygen present (Havaux et al., 2000; Mittler, 2002; Trebst, 2003). On the other hand, α-tocopherol scavenges singlet oxygen in cooperation with carotenoids and prevents the propagation of lipid peroxidation in photosynthetic membranes (Trebst, 2003; Falk and Munné-Bosch, 2010). Furthermore, some Labiatae species, such as rosemary and sage, present additional mechanisms of photoprotection, including the accumulation of the phenolic diterpene and carnosic acid in chloroplasts (Munné-Bosch and Alegre, 2000, 2003; Peñuelas and Munné-Bosch, 2005). Carnosic acid is a potent antioxidant that, following oxidation, gives rise to carnosol and other oxidized abietane diterpenes with lactone structures, including rosmanol, isorosmanol and dimethyl isorosmanol. Therefore, the oxidation of carnosic acid has been suggested as a reliable way of measuring the redox state of chloroplasts in plants (Munné-Bosch and Alegre, 2003).

Under salt-stress conditions, toxic ions (Na^+ and Cl^-) preferentially accumulate in the older, lower leaves, which is a fairly common salt-tolerance mechanism in several glycophytes (Greenway and Munns, 1980). Nonetheless, considerable amounts of toxic ions also accumulate in the upper leaves when the plant is exposed to increasing salt concentrations in the medium. Thus, the younger, upper leaves are simultaneously exposed to water deficit, ion toxicities and high light stress, while the older, lower leaves usually require a lower photoprotective demand (Niinemets and Kull, 2001; Baldocchi et al., 2002; Niinemets and Valladares, 2004). It has been shown that the sun leaves of *Fagus sylvatica* and *Quercus petraea* display larger pools of zeaxanthin, ascorbate and glutathione and exhibit higher antioxidant enzyme activities than shade leaves (Hansen et al., 2002). Similarly, the tocopherol content per unit of chlorophyll was reported to increase in sun *versus* shade leaves in *F. sylvatica* (Lichtenthaler, 1971; García-Plazaola et al., 2000; García-Plazaola and Becerril, 2001) and *Quercus ilex* (García-Plazaola et al., 1999), which suggests that their content changes in response to the photoprotective demand. Therefore, we hypothesized that the photoprotective demand and antioxidant protection of the upper and lower canopy leaves might differ in plants exposed to increasing NaCl concentrations, an aspect that so far has not been investigated.

Common sage (*Salvia officinalis* L.) is an aromatic plant used in the pharmaceutical and food industries for its richness in essential oils, flavonoids and phenolic diterpenes. It is well known as a moderately drought-resistant plant and is believed to be a moderately salt-resistant glycophyte (Maas and Hoffman, 1977; Tounekti et al., 2010). Under drought conditions, the plant activates several mechanisms of photo- and antioxidant protection (Munné-Bosch and Alegre, 2000). However, little is known about how these mechanisms alter in response to salt stress in this species, or how leaf position in the canopy affects this response. In the present study, we examined: (i) the extent to which sage plants can withstand salt stress; (ii) what mechanisms are involved in salt-stress tolerance; and (iii) how leaf position in the canopy determines the photoprotective demand, photo-oxidative stress and activation of mechanisms of photo- and antioxidant protection in salt-stressed plants.

2. Materials and methods

2.1. Plant material and salt treatments

One-year-old rooted cuttings of *S. officinalis* plants were grown in 5-L plastic pots containing desert dune sand in a glasshouse covered with a shade net. Plants were irrigated every 4 days with a complete nutrient solution with an initial total ion concentration of 4.5 mM and electrical conductivity (EC) of 3.04 dS m^{-1} (consisting of N, 1.8 mM; P, 0.35 mM; K, 0.64 mM; Ca, 1.0 mM; Mg, 0.35 mM; S, 0.35 mM; Fe, 0.03 mM; Zn, 0.4 μM; Mn, 5.0 μM; Cu, 0.1 μM and B, 0.02 mM). Salt stress was imposed on plants by increasing the amounts of NaCl up to 50 mM (low salt dose, LS) and 100 mM (high salt dose, HS). To avoid osmotic shock, NaCl concentrations were gradually increased by 25 mM per day until the desired concentrations were reached. Each pot received about 400 mL of the solution, enough to start draining through the base. Throughout the treatment period, the EC of the culture medium was kept at approximately 15.0 dS m^{-1} and 9.0 dS m^{-1} for 50 and 100 mM NaCl treatments, respectively, by washing the substrate twice with tap water before applying the new nutrient solution. Two experiments were carried out with two separate sets of plants during July and August 2007 with maximum and minimum air temperatures ranging from 29 °C to 33 °C and from 20 °C to 25 °C, respectively, while relative air humidity ranged from 43% to 60%. Maximum daily PPFD ranged from 1000 to 1100 μmol m^{-2} s^{-1} throughout the experiment.

For the time-course evolution experiment (Experiment 1), a batch of four plants of each treatment (50 mM and 100 mM NaCl) was sampled after 0, 2, 3, 4 and 5 weeks of adding salt. Pools of leaves taken from different positions in the canopy (including leaves from the upper, middle and lower parts of the canopy in similar amounts) were collected from each plant between 9 and 11 a.m. local time, immediately frozen in liquid nitrogen and then stored at −80 °C for biochemical analyses.

For the leaf canopy position experiment (Experiment 2), four plants of each treatment were sampled after 5 weeks of adding salt between 9 and 11 a.m. local time. The leaves were subdivided into two types: (i) young, upper leaves (three to four nodes from the apex), which were around 6 months old and received a maximum daily solar radiation of 1000–1100 μmol m^{-2} s^{-1} (50% of the full sunlight), and (ii) older, lower leaves (non-senescent leaves from the upper part), which were around 12 months old and received 600–750 μmol m^{-2} s^{-1} (35% of the full sunlight). These leaves were collected between 9 and 11 a.m. local time, immediately frozen in liquid nitrogen and then stored at −80 °C for biochemical analyses.

2.2. Growth, water relations, ion contents and gas exchange

Fresh mass, leaf area and number of leaves per plant were used to estimate plant growth. Leaf area was measured using a portable leaf area metre (AM300 Portable Leaf Area Meter, ADC BioScientific Ltd, Great Amwell, UK). The relative water content (RWC) and the water content (WC) of the leaves were calculated using the following equations: $RWC = 100 \times [(FW - DW)/(TW - DW)]$ and $WC = (FW - DW)/DW$, where FW is the fresh weight, TW is the turgid weight after re-hydration in the dark for 20 h, and DW is the dry weight after oven-drying the leaves at 80 °C for 48 h. The predawn water potential (Ψ_w) of the shoots was measured with a pressure chamber (Soil Moisture Equipment Co., Santa Barbara, CA, USA). For determination of ion contents, 1 g of dried, ground leaves was extracted with 20 mL of 0.1 M HNO_3. After filtration,