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An integrated approach to understand the mechanisms underlying salt stress tolerance in *Casuarina glauca* and its relation with nitrogen-fixing *Frankia* Thr

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Abstract Salinity is one of the most wide spread abiotic stresses affecting agricultural productivity, with an impact on more than 800 million hectares worldwide. A promising solution for the recovery of saline soils encompasses the use of actinorhizal plants, a group of perennial dicotyledonous angiosperms including species highly resilient to extreme environmental conditions. These plants are able to establish root-nodule symbiosis with N_2-fixing actinobacteria of the genus *Frankia*. In this review, we discuss the main physiological and biochemical mechanisms underlying salt tolerance in the model *Casuarina glauca* supplemented with chemical nitrogen or obtaining it from symbiotic *Frankia*. In the first part, an overview of the impact of increasing NaCl concentrations in photosynthesis, antioxidative system and membrane integrity is presented. The second part addresses the effect of salt stress in the symbiosis between *C. glauca* and *Frankia* strain Thr. Preliminary results from analyses of the branchlets proteome and nodule metabolome are presented as well.

Keywords *Casuarina glauca* · *Frankia* · Membrane integrity · Oxidative stress · Photosynthesis · Salt stress

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1 Introduction

The model actinorhizal plant *Casuarina glauca* Sieb. Ex Spreng. (family *Casuarinaceae*) is native to the south east coast of Australia (Ganguli and Kennedy 2013). This species has been introduced to and is now widely distributed in the tropical and sub-tropical regions of America (USA, Mexico, Bolivia, Puerto Rico, Bahamas and Florida), Africa (Egypt, Kenya, South Africa, Malawi and Mauritius), Asia (Iran, India, Indonesia, Israel and Malaysia) and New Zealand (Zhong and Zhang 2003; He and Critchley 2008). As an actinorhizal plant, *C. glauca* establishes a nitrogen-fixing root-nodule symbiosis with *Frankia* bacteria. It is highly resilient to extreme environments, being commonly found in saline soils of the coastal zones and used to restore marginal soils and to prevent desertification (Diagne et al. 2013).

Considering that salinity is expected to affect more than 50 % of all arable land by the year 2050 (Wang et al. 2003), the study of the mechanisms underlying salt tolerance in plants is a priority in agro-forestry research. Under this context, we have used a multidisciplinary approach to analyse the impact of salinity in *C. glauca* as well as to determine its limits of salinity tolerance and the contribution of symbiotic *Frankia* to this ability. For that, *C. glauca* plants (ca. 1.5 year-old) nodulated by *Frankia* Thr (named NOD^+) or supplied with KNO_3 (named KNO_3^+) were exposed to increasing concentrations of NaCl, i.e. 200, 400 and 600 mM as described in

Batista-Santos et al. (2015). The impact of the imposed stress was then evaluated at the morphological, physiological and biochemical level in order to determine the major changes in the photosynthetic pathway, antioxidative system and membrane stability, which are amongst the first stress metabolic and structural "targets". In parallel, the effect of salt on N_2-fixation activity was also determined. Complementary, an "omics" approach was applied based on the analysis of the branchlets proteome and nodule metabolome. In this review we present the key processes and molecules underlying salt tolerance in *C. glauca*.

2 Impact of salinity in branchlets

2.1 Changes in morphology, water and mineral content

C. glauca plants were able to survive above seawater levels of salinity (up to 600 mM NaCl). However, with stress progression up to such extreme salinity conditions, phenotypic changes, i.e. branchlets chlorosis and necrosis were observed, accompanied by a decrease in growth (Batista-Santos et al. 2015). Additionally, Na^+ and Cl^- accumulated in the branchlets of both NOD^+ and $KNO_3{}^+$ plants. Despite this, the relative water content (RWC) was only moderately reduced (ca. 10 % at 600 mM NaCl) and concomitant with a strong decrease in osmotic potential ($\Psi\pi 100$), reflecting an osmotic adjustment that allowed the plants to maintain, at least in part, a potential gradient of water influx, and to sustain metabolic activity (Chaves et al. 2009). Indeed, the synthesis of compatible solutes for osmotic adjustment is an acclimation response to salinity (Kozlowski 1997), which also facilitates adjustments in ion exchange (uptake, extrusion and sequestration) and promotes the re-equilibrium of cellular homeostasis, detoxification and survival under stress (Chaves et al. 2009). Besides that, the N-contents were quite stable (15–20 mg g^{-1} DW) in both plant groups, suggesting that under rising NaCl concentrations the plants entered in a new equilibrium with gradual growth reduction.

2.2 Carbon assimilation tolerance at the photochemical level

Net photosynthesis (P_n) and stomatal conductance to water vapour (g_s) were strongly reduced with increasing salinity in both nodulated and non-nodulated groups, reaching values close to 10 % as compared to control non-stressed plants (Batista-Santos et al. 2015). This might be related to stomatal closure, one of the first effects of stress conditions, resulting in the down-regulation of the photosynthetic pathway (Chaves et al. 2009). However, despite the strong reduction in g_s, the impact of salt on P_n might be attributed to metabolic changes as the internal CO_2 concentration (C_i) was comparable to that of the unstressed control group (0 mM NaCl). On the other hand, the photosynthetic assimilation potential (A_{max}) was much less affected than P_n, with reductions of ca. 30 % at 600 mM NaCl (vs. 90 % in P_n) in both NOD^+ and $KNO_3{}^+$ plants. This observation, together with the strong increase of Na^+, suggests that the effects on P_n are largely reversible. Accordingly, most of the fluorescence parameters were not affected by the presence of salt, but some non-stomatal limitations associated with photosystem (PS) II, electron transport and enzyme activity were observed, particularly at 400 and 600 mM NaCl. This observation was consistent with the growth analysis, as well as with RWC and A_{max} trends, supporting the hypothesis that the salt tolerance capacity of *C. glauca* is linked to photosynthetic adaptations. In fact, the energy driven to photochemical events (given by the photochemical quenchings, q_L and q_P) was nearly maintained in both plant groups with increasing salt concentrations, which might be attributed to the strong tolerance of photochemical events (Ramalho et al. 2014a). Moreover, the photochemical efficiency of PSII under photosynthetic steady-state conditions (F_v'/F_m') gradually decreased whereas non-photochemical quenching of the excited state of chlorophyll *a* (NPQ) followed the opposite trend.

Altogether, the data suggest that the loss of photochemical efficiency might reflect energy dissipation mechanisms that compete for light energy, rather than being a response to damaging events. This effect might be associated with the increased synthesis of zeaxanthin and antheraxanthin (Batista-Santos et al. 2015), and is consistent with the lower impact on A_{max} than on P_n, with the variation in quantum yield of non-regulated energy dissipation of PSII (Y_{NPQ}) and quantum yield of down-regulated energy dissipation of PSII (Y_{NO}) (see below), as well as with the maintenance of a significant degree of functioning potential of the photosynthetic machinery (Ramalho et al. 2014a).

Although relevant impairments were detected in the estimate of quantum yield of photosynthetic non-cyclic electron transport (Y_{II}) at 400 mM NaCl, strong effects on the electron transport rates (ETR) and on in vivo activities of PS I and II were only observed at 600 mM NaCl, suggesting that the structures supporting thylakoid electron transport represent one of the less salt-sensitive targets (Sudhir and Murthy 2004). Considering that Y_{II}, q_L, and q_P suffered a much lower impact than P_n, and that the potential electron transport was considerably maintained (at least until 400 mM NaCl), some energy use could have occurred through photorespiration and/or partitioning of electrons to oxygen (although reactive O_2 forms must be kept under strict control by complementary antioxidative mechanisms), thus contributing to maintain PSII activity.

Similar to NPQ, the regulated energy dissipation of PSII (Y_{NPQ}) showed a gradual increase, reflecting a reinforcement of the quantum yield of non-photochemical energy loss in PSII due to the down-regulation of the light-harvesting

function (Kramer et al. 2004). This effect is usually associated with photoprotective mechanisms such as high-energy quenching (q_E), protonation of PsbS (a protein associated with the PSII reaction centre), or accumulation of zeaxanthin (Li et al. 2000). Furthermore, the non-regulated energy (heat and fluorescence) dissipation of PSII (Y_{NO}) did not change even at 600 mM NaCl. According to Huang et al. (2011), Y_{NO} is usually stable, but high values reflect limitations in the use of the incident radiation. Therefore, when the yield of photochemistry (Y_{II}) decreased in *C. glauca*, it was compensated by the enhancement of down-regulated processes (Y_{NPQ}) instead of non-photochemical losses related to photo-damaging effects (Y_{NO}). This indicates that *C. glauca* plants were able to trigger photoprotective mechanisms, thus limiting severe impairments in the photosynthetic apparatus (Kramer et al. 2004; Huang et al. 2011).

2.3 Biochemical sensitivity to salt

In contrast with branchlets photochemistry, salt stress on *C. glauca* had a strong impact at the biochemical level, namely on key enzymes from the photosynthetic and respiratory pathways (Batista-Santos et al. 2015). In fact, at 600 mM NaCl the initial and total activities of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) decreased sharply in both plant groups (down to ca. 60 %-80 % of control levels). Such reduction seems to be linked to enhanced degradation and/or reduced synthesis, rather than to an impact on the activation state, which was fairly well maintained. A similar effect was also observed for ribulose 5-phosphate kinase (Ru5PK). Nevertheless, it should be underlined that the NaCl effects on photosynthetic enzymes and in the PSs were quite moderate until 400 mM NaCl confirming a strong salt tolerance capacity of *C. glauca* (Jaoudé et al. 2013).

The activities of the respiratory enzymes pyruvate kinase (PK) and NADH-dependent malate dehydrogenase (MDH) were two of the most sensitive parameters, being reduced to residual values (< 15 %) at 600 mM NaCl (Batista-Santos et al. 2015). Thus, it is likely that in *C. glauca* the negative impact of salt on the respiratory enzymes affects the respiratory metabolism which therefore will not be able to constitute an alternative source of energy (Kozlowski 1997).

Regarding the antioxidant response, high activities of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and catalase (CAT) were observed from 200 mM NaCl upwards in both KNO_3^+ and NOD^+ plants (unpublished data). The overproduction of Reactive Oxygen Species (ROS) at the PSs level is promoted by stress conditions that reduce the photochemical use of energy (Asada 1994; Logan 2005). Thus, the control of ROS detoxification is crucial to avoid cell damage e.g. lipid peroxidation, inactivation of enzymes and PSs as well as the degradation of pigments, protein and DNA (Foyer 2002; Smirnoff 2005). Therefore, the control of oxidative stress through the reinforcement of ROS scavenging- and detoxifying mechanisms is crucial to plant stress tolerance and survival (Logan 2005; Smirnoff 2005; Fortunato et al. 2010). In this way, the observed reinforcement of antioxidant enzymes in *C. glauca*, might have contributed to the maintenance of potential photochemical performance (Batista-Santos et al. 2015).

2.4 Non-structural carbohydrates

Sugars and sugar alcohols are often involved in plant response to dehydration as osmoprotective molecules that help to preserve biological functions (Kozlowski 1997; Chaves et al. 2009). Despite a moderate increase of sucrose and arabinose with increasing salt concentrations, osmotic adjustment in *C. glauca* shoots was not associated to the accumulation of soluble sugars (Batista-Santos et al. 2015). Instead, the increase in sucrose levels seems to reflect a decreased export from source tissues as well as a decreased use in sink tissues. This might be explained by the limited water and solute flow within the plant due to low g_s resulting from stomatal closure, and to a decline of sink demand-driven growth inhibition (Ramalho et al. 2014a). The latter might also explain the increase in starch. These data are in agreement with the severe reduction in glucose and fructose levels. Raffinose family oligosaccharides (RFOs) and sugar alcohols do not seem to be involved in the salt response of *C. glauca*, as only small quantities were detected (Batista-Santos et al. 2015).

2.5 Membrane integrity and composition

Membrane permeability remained practically unchanged in both KNO_3^+ and NOD^+ plants from 0 to 400 mM NaCl and leakage was only evident at 600 mM NaCl (Scotti-Campos et al. 2016). This result is another indicator of the high salinity tolerance in *C. glauca* and is in line with the moderate impact of salt stress on photosynthetic structures and performance (Batista-Santos et al. 2015). On the other hand, both symbiotic and non-symbiotic plants presented a similar pattern of variation in the amounts of total and individual fatty acids (FA), with reductions of ca. 50 % at 400 and 600 mM NaCl. Nevertheless, the degree of membrane unsaturation was quite stable at 200 and 400 mM NaCl, probably due to the triggering of antioxidative mechanisms that controlled lipoperoxidation (Ramalho et al. 2014b). This, together with the low dehydration (up to 20 % at 600 mM NaCl) might have prevented the loss of cellular turgor, contributing to the preservation of membrane integrity and functioning, a crucial feature for plant survival under stress (Batista-Santos et al. 2015).

2.6 Response of the branchlets proteome

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) led to the identification of a set of 357 proteins, from which 127 and 82 were differentially expressed along the stress imposition in KNO_3^+ plants (Fig. 1a) and NOD^+ plants (Fig. 1b), respectively. In a preliminary analysis, stress-responsive proteins were categorized into three functional groups: ion transport (ca. 15 % in KNO_3^+ and 10 % in NOD^+); metabolic and cellular processes (ca. 60 % in KNO_3^+ and 70 % in NOD^+); and response to stimulus (ca. 25 % in KNO_3^+ and 20 % in NOD^+). In KNO_3^+ plants, changes in protein levels were residual at 200 mM NaCl (ca. 5 %), increasing to ca. 30 % at 400 mM NaCl and to ca. 85 % at 600 mM NaCl. This observation is line with the morphological, physiological and biochemical analysis, reflecting the strong capacity of *C. glauca* to cope with salt stress. NOD^+ plants contained a higher amount of differentially expressed proteins than KNO_3^+ plants at the two first stress levels (ca. 15 % at 200 mM NaCl and ca. 50 % at 400 mM NaCl), while at 600 mM the percentage was lower (ca. 60 %). These differences are probably linked to the fact that at 200 mM NaCl NOD^+ plants are double-stressed since at this salt concentration the symbiosis is turned to residual levels, thereby abolishing nitrogen supply (Duro et al. 2016).

3 Impact of salt stress in the *C. glauca-Frankia* Thr symbiosis

3.1 Nitrogen assimilation

According to Duro et al. (2016), symbiotic nitrogen fixation in nodules induced by *Frankia* strain Thr on roots of *C. glauca* was reduced to residual levels at 200 mM NaCl. At this salt concentration both bacterial *nifH* mRNA (encoding nitrogenase reductase) and nitrogenase activity showed a sharp decrease in nodules, i.e. 15 % and 5 % of the control values, respectively. At 600 mM NaCl the activity values were down to 2 % and 1 %, respectively. This was accompanied by a strong impairment of the transcriptional activity of plant genes involved in the ammonium assimilation pathway, namely glutamine synthetase (*CgGS*) that binds ammonia to glutamate, yielding glutamine (McNally and Hirel 1983; Cramer et al. 2002), and asparagine synthase (*CgAS*) that uses glutamine to transamidate aspartate resulting in the formation of asparagine and glutamate (Sieciechowicz et al. 1988; Cramer et al. 2002). Concomitantly, the expression of plant symbiotic genes involved in the control of *Frankia* infection, *CgDEF* (encoding a defensin; Hocher et al. 2011) and *Cg12* (encoding a subtilisin-like protease; Laplaze et al. 2000) were also down-regulated with increasing NaCl levels, which might reflect the inactivation of bacterial nitrogen fixation or a block in the infection of nodule cells by bacteria (Duro et al. 2016).

3.2 GC-MS metabolite profiling of the nodule metabolome

Gas chromatography coupled to mass spectrometry-based metabolomics approaches (GC-MS) performed on extracts from nodules detected a set of 32 primary metabolites (Fig. 2). Among these, amino acids were the most abundant metabolites with a total of 17 chemical species. Some classes of osmolytes such as sugars and sugar-alcohols (e.g., glucose, fructose, raffinose, galactinol, and myo-inositol) were also found. As mentioned above, osmolytes are known to be very important under water stress conditions because they not only sustain cell turgor by osmotic adjustment and stabilize enzymes, but also confer protection against oxidative damage by being involved in ROS scavenging or in the suppression of ROS production to consequently help re-establish the cellular redox balance (Mittler 2002; Yancey 2005; Slama et al. 2015). According to the GC-MS metabolite profiling, the well-known osmolytes proline, sucrose, trehalose, and mannitol remained unchanged in the nodules of *C. glauca* under increasing salt concentrations (Fig. 2). Similar observations were made for branchlets (Batista-Santos et al. 2015). These

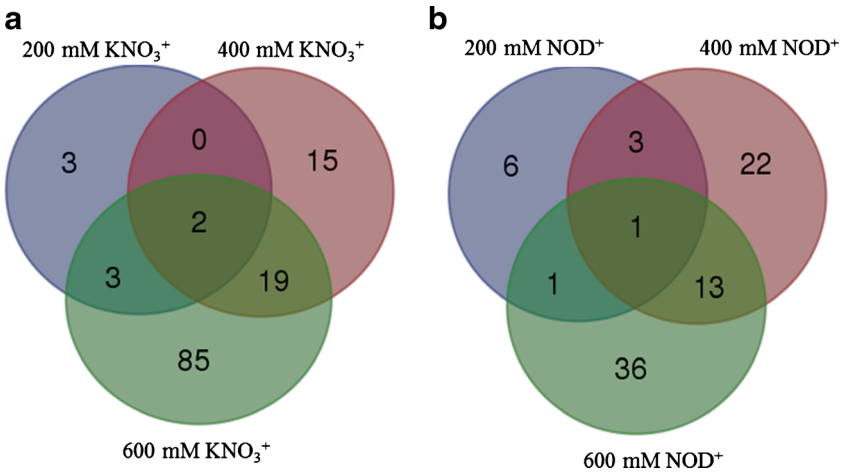

Fig. 1 Venn Diagrams indicating the number of differentially expressed proteins in branchlets of **a** non-symbiotic ($KNO3^+$) and **b** symbiotic (NOD^+) *Casuarina glauca* plants

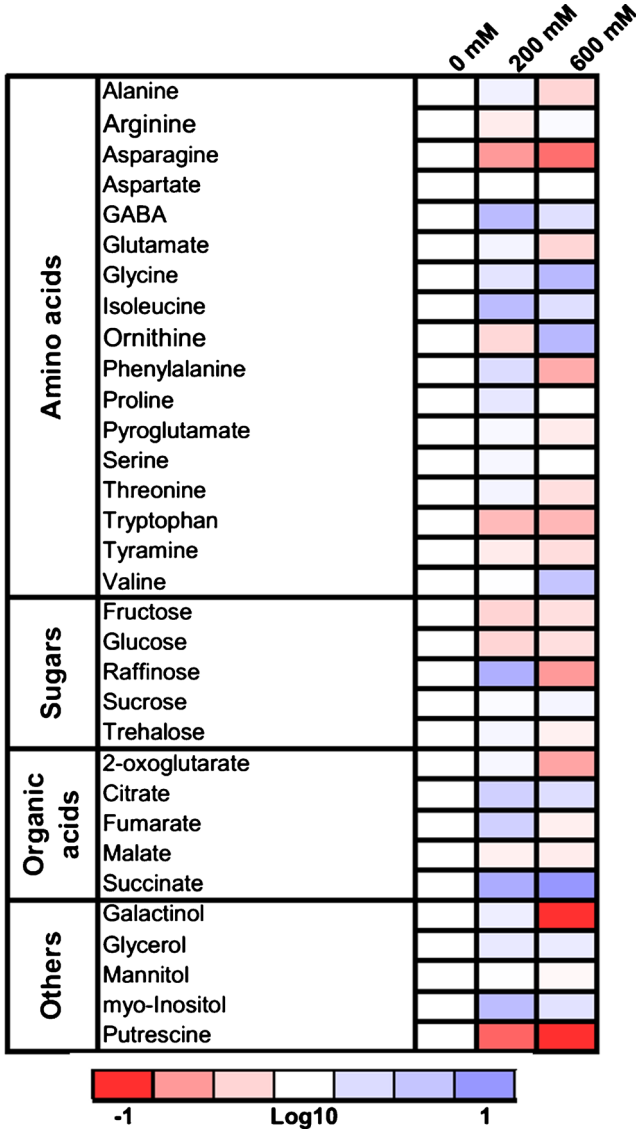

Fig. 2 Metabolite profile of *C. glauca* nodules exposed to 200 and 600 mM NaCl

results suggest that such compounds do not contribute to the osmotic adjustment under salt stress conditions. On the other hand, contrarily to what was observed in branchlets (see 2.4), raffinose may be involved in osmoprotection in nodules as its levels increased significantly at 200 mM NaCl (Fig. 2). At 600 mM NaCl, the levels of raffinose decreased considerably which is probably due to the fact that at this NaCl concentration the overall plant metabolism suffers a clear negative impact (Batista-Santos et al. 2015).

4 Data integration

The capacity of *C. glauca* to tolerate levels of salinity above that of seawater is associated with a series of physiological and biochemical adjustments that allow the plant to overcome stress. First, *C. glauca* shows remarkably low tissue dehydration and significant osmotic adjustments that minimize the impact of salt on cell water content and the photosynthetic machinery. Thus, despite the reduction of P_n to almost negligible values, the photosynthetic assimilation potential (A_{max}) is maintained at unexpectedly high values in the presence of salt, reflecting the maintenance of the global potential of photosynthetic functioning. Indeed, the photosynthetic limitations observed until 400 mM NaCl seem to be associated with the down-regulation of processes rather than severe damage. On the other hand, the impact of stress on key photosynthetic and respiratory enzymes suggests that salt stress induces biochemical damage rather than photochemical impairments. Second, salt stress tolerance in *C. glauca* is related to the preservation of membrane stability as well as the maintenance of a controlled oxidative state environment. Finally, the ability of *C. glauca* to endure high NaCl concentrations seems to be innate and uncoupled from the presence of the symbiotic *Frankia* strain Thr. Indeed, even under N-depleted conditions, that is, in NOD^+ plants from 200 mM NaCl upwards, when the symbiosis was no longer functional, *C. glauca* was able to survive in the presence of high levels of salt. In the near future we expect to complete the "omics" approach to understand the overall picture of salt stress tolerance mechanisms in *C. glauca*. To our knowledge, only few reports on this topic are available, and most of these studies were not broad enough to access the mechanisms underlying stress tolerance in actinorhizal plants.

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