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Improving salt stress responses of the symbiosis in alfalfa using salt-tolerant cultivar and rhizobial strain

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ABSTRACT

Salt stress can affect alfalfa growth directly by adversely affecting metabolism, or indirectly by its effect on *Rhizobium* capacity for symbiotic N_2 fixation. Growth and carbohydrate metabolism in leaves, roots and nodules of two alfalfa cultivars (*Medicago sativa* cv Apica and salt-tolerant cv Halo) in association with two rhizobial strains (A2 and salt-tolerant Rm1521) exposed to different levels of NaCl (0, 20, 40, 80 or 160 mM NaCl) were assessed under controlled conditions. For both cultivars, shoot and root biomasses and shoot to root ratio significantly declined with increasing NaCl concentrations. Under 80 mM NaCl, Halo plants yielded 20% more fresh shoot biomass than Apica while plants inoculated with Rm1521 allocated more biomass to the roots than to the shoots compared to A2. Halo plants maintained a steady shoot water content (about 80%) under the entire range of NaCl concentrations. Shoot water content was more variable in Apica. Apica in association with salt-tolerant strain Rm1521 maintained a better water status than with strain A2, as indicated by the higher shoot water content at 80 mM NaCl. Under salt stress, two major compatible sugars involved in plant osmoregulation, sucrose and pinitol, increased in leaves while a large accumulation of starch was observed in roots. In nodules, pinitol, sucrose and starch increased under salt stress and were much more abundant with strain Rm1521 than with A2. This suggests that there could be an active transport from the shoot to the nodules to help maintain nodule activity under NaCl stress and that strain Rm1521 increases the sink strength toward nodules. Our results show that combining cultivars and rhizobial strains with superior salt tolerance is an effective strategy to improve alfalfa productivity in salinity affected areas.

1. Introduction

Salt stress is one of the most important abiotic factors limiting plant growth and productivity especially in arid and semi-arid regions. In Canada, this occurs extensively in the Prairie Provinces, where 79% of Canada's agricultural lands are located. The total extent of moderate-to-severe salinity in the Prairies, resulting in a 50% reduction in productivity, was estimated to be around 1.4 million hectares (Eilers et al., 1997).

Plants tend to cope with salt stress by different adaptation strategies including physiological, biochemical, and molecular mechanisms (Bohnert et al., 1995; Munns and Tester, 2008) that facilitate retention and/or acquisition of water, protect chloroplast functions and maintain ion homeostasis (Parida and Das, 2005). Biochemical mechanisms include accumulation of compatible solutes such as amino acids, sugars and polyols (Ford, 1984). These organic solutes are able to accumulate at high concentration in the cytoplasm, contributing to turgor maintenance and protecting enzymes and other cellular structures against damage by ions or dehydration (Bartels and Sunkar, 2005). Plant species or cultivars greatly vary in their tolerance to salt (Noble et al., 1984; Al-Khatib et al., 1994; Munns, 2002) and thus, differ in their capacity to remain productive as soil salinity increases. Alfalfa is the most important forage crop species in Canada where it is the third largest crop by area. It is cultivated over 4.5 million hectares in the Canadian Prairies accounting for 76% of the total national production area of pure alfalfa or alfalfa mixtures (Statistics Canada, 2012). Alfalfa is considered as moderately tolerant to salt and can tolerate an equivalent of 20 mM NaCl. Alfalfa with improved salt tolerance has been developed using conventional

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breeding and genetic engineering approaches (Bohnert and Jensen, 1996; Flowers, 2004). High salinity may also affect alfalfa performance by reducing the efficiency of the *Rhizobium*–legume symbiotic N_2 fixation (Bernstein and Ogata, 1966; Delgado et al., 1993; Zahran, 1999). It has been established that *Rhizobia* are, in general, more tolerant to salt stress than the host plant, but rhizobial strains greatly vary in growth and survival under salt stress (Soussi et al., 1998; Swaraj and Bishnoi, 1999). It has been suggested that a combination of stress-tolerant cultivars and stress-tolerant *Rhizobia* may result in synergistic advantages in the ability of legumes to grow and survive under saline conditions (Hashem et al., 1998; Nogales et al., 2002). This approach has been successfully used for improving biological nitrogen fixation under salt stress of soybean (*Glycine max*) (Elsheikh and Wood, 1995), *Acacia ampliceps* (Zou et al., 1995) and *Phaseolus vulgaris* (Nogales et al., 2002). Mohammad et al. (1989) showed that salt stress-tolerant *Medicago sativa* plants exhibited higher drought tolerance when inoculated with a salt stress-tolerant strain of *Sinorhizobium meliloti*.

In the present study, we investigated the mechanisms of alfalfa tolerance to salt stress. Our hypothesis was that tolerant symbiotic partners would act in synergy to alleviate salt stress in alfalfa. To reach this goal, we measured the concentration of carbohydrate compounds typically associated with stress tolerance in leaves, roots and nodules of alfalfa under five levels of NaCl. We compared the response of one salt-sensitive and one salt-tolerant cultivar in association with one salt-sensitive and one salt-tolerant rhizobial strain.

2. Materials and methods

2.1. Plant material

This study was carried out on two cultivars of alfalfa (*M. sativa* subsp. *sativa*), Apica and Halo. Apica was developed at the Agriculture and Agri-Food Canada Research Station in Québec City (Québec, Canada) and was tested across Eastern Canada for its yield, persistence and winter survival (Michaud et al., 1983). Halo is a synthetic variety with 192 parent plants selected sequentially for germination, seedling growth, and mature plant re-growth, as well as salinity tolerance (Steppuhn et al., 2012).

2.2. Inoculum production

Two strains of *S. meliloti* were used, A2 (Balsac) isolated from Eastern Canada (Bordeleau et al., 1977) and Rm1521, a salt-tolerant strain isolated from the Ottawa vicinity (Bromfield et al., 1994). Rhizobial strains were grown on yeast mannitol agar plates (Vincent, 1970). Each strain was resuspended in yeast extract mannitol broth, and then placed in a shaking incubator (120 rpm, Lab-Line Orbit Environ-shaker, Melrose Park, IL) at 28 °C for 1 week. Viability counts were then performed and the inoculum was adjusted to 10^9 cells mL^{-1}.

2.3. Plant growth conditions

Alfalfa seeds were surface-sterilized by immersion in ethanol 95% for 30 s, and in 5% NaClO for 10 min, and then washed five times with sterile water. Sowing was performed in 20 cm diameter, 20 cm deep plastic pots filled with turface (Profile Products LLC, Buffalo Grove, IL). For each cultivar, twenty seeds were sown per pot: two seeds were placed in each of ten small cavities in which 200 μL of inoculum was added. Half of the pots were inoculated with A2 and the other half with Rm1521 inoculum.

Plants were grown in a large growth room under a 21/17 °C day/night temperatures regime, a 16 h photoperiod and a photosynthetic photon flux density of 600–800 μmol photons $m^{-2} s^{-1}$ provided by a mixture of high pressure sodium and metal halide 400 W lamps (PL light Systems, Beamsville, ON, Canada). After one week, seedlings were thinned to ten plants per pot and a second inoculation was done using 200 μL of inoculant per seedling. Plants were fertigated daily with 0.25X N-free nutrient solution during the first week and with 0.5X N-free nutrient solution during the second week (1X nutrient solution contained 111.8 mg $P L^{-1}$, 141.1 mg $K L^{-1}$, 2.100 mg $Fe L^{-1}$, 0.600 mg $Mn L^{-1}$, 0.120 mg $Zn L^{-1}$, 0.030 mg $Cu L^{-1}$, 0.390 mg $B L^{-1}$, 0.018 mg $Mo L^{-1}$, 48.62 mg $Mg L^{-1}$, and 0.952 mg $Co L^{-1}$). Two weeks after sowing, plants were fertilized once with N (2 mM N + 0.5 X nutrient solution). Salt stress treatments consisted of 0, 20, 40, 80, or 160 mM NaCl which were applied on 3 week-old plants. To avoid osmotic shock, NaCl concentrations were gradually increased during one transition-week starting on day 1 with 20 mM NaCl for all NaCl-treated pots. NaCl concentration was then doubled every two days until final concentrations were reached. Once the targeted salt stress levels were reached, plants were fertigated daily with 0.5X nutrient solution containing the appropriate NaCl concentration. Salt stress was applied for six weeks and then plants were sampled.

2.4. Plant biomass measurements and tissue sampling

At sampling, plants were carefully removed from substrate and gently washed under tap water. Excess water was removed by gently pressing roots in absorbent paper towels. Roots were cut from shoots, and root and shoot fresh weights (FW) were recorded. To reduce within-pot variation, pooled subsamples of shoots and roots from three plants were dried at 55 °C to a constant weight for total dry weight (DW) and water content assessments. For the remaining plants, shoots were separated into leaves and stems while nodules were detached from roots.

Subsamples of leaves, roots and nodules were lyophilized prior to grinding for biochemical assessments. Nodule samples were ground using a Tissue Lyser (Tissue Lyser II, Retsch, QiaGen) while other tissues were ground using a mixer mill (Mixer Mill 301, Retsch Inc.).

The relative biomass curve response to salt stress was established for shoot and root biomass using the following equation:

$$\text{Relative biomass}(\%) = \frac{\text{biomass yield under NaCl stress}}{\text{biomass yield at0mM NaCl}} \times 100$$

2.5. Extractions and analyses of carbohydrates

Lyophilized samples of leaves and roots (0.2 g) were extracted in 7 mL and nodules (0.1 g) were extracted in 4 mL of deionized water. Tubes were heated 20 min at 65 °C to stop enzymatic activity. Tubes were kept overnight at 4 °C for optimal extraction and then homogenized on a vortex mixer and centrifuged 10 min at 3000 rpm at 4 °C (Bertrand et al., 2003). 1 mL subsample of supernatant was transferred to 1.5 mL microtubes and centrifuged for 3 min at 13,000 × *g* prior to HPLC analyses. The non-soluble residues left after extraction were washed twice with 10 mL of methanol and used for starch determination.

Mono-, di-, tri- and tetra-saccharides were separated and quantified on a Waters analytic system controlled by the Empower II software (Waters, Milford, MA, USA). Sugars were separated on a HPX-87P column (Bio-Rad) at 80 °C with a flow rate of 0.5 mL min^{-1} with water. Peak identity and quantity were determined for sucrose, glucose, fructose and pinitol by