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Variable salinity responses and comparative gene expression in woodland strawberry genotypes

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ABSTRACT

Commercial strawberry cultivars are sensitive to salinity. These plants are octoploid (2n = 8x = 56) making analysis of complex traits such as salt tolerance difficult. Hence, the objective of this study was to evaluate eight woodland diploid strawberry genotypes for their physiological and genetic responses under high salinity conditions. Shoot salt tolerance (ST) index of eight genotypes irrigated with water of electrical conductivity (EC_{iw}) of 8 dS m^{-1} ranged from 0.41 to 0.86. Average Cl concentration in leaves was more than 11-fold higher than Na concentration, indicating that Cl^- toxicity is more critical than Na^+ for diploid strawberries. Improved water use efficiency (*WUE*) due to reduced stomatal conductance (*gs*), intercellular CO_2 concentration (*Ci*), and transpiration rate (*Tr*) was associated with improved ST index. Expression analyses of eleven genes involved in Na^+ and Cl^- transport revealed differences in component traits responsible for salt tolerance. Increased expressions of *SOS2, NHX1* and *NHX2* in salt-tolerant genotypes suggest that Na^+ sequestration in vacuoles may play an important role in salt tolerance in strawberry. Additionally, a chloride channel gene (*CLC_C*) was shown to be critical for salt tolerance. Understanding the genetic mechanisms regulating salt tolerance will be critical in developing strawberry germplasm more tolerant to salinity.

1. Introduction

Salinity is one of the major limiting factors for agriculture and is an increasing problem in drought-prone regions of the world (Munns and Tester, 2008). About 1/5th of the irrigated land used for agriculture is affected by salt. In addition, the use of poor quality (recycled) water for irrigation increases the exposure of crops to salinity. Salt tolerance is directly related to plant growth in the presence of a saline environment and can be measured in terms of survival, maintenance of vigor and yield (Munns and Tester, 2008). However, morphological appearance observed in response to salinity displays only a piece in the puzzle. Physiological, biochemical and genetic determinants and their relationship must be understood to determine the most important aspects involved in salinity tolerance.

Strawberry is a sensitive crop with a low soil electrical conductivity (EC_e) threshold (EC_e = 1.0 dS m^{-1}) when compared with crops such as tomato (EC_e = 2.5), artichoke (EC_e = 6.1), cowpea (EC_e = 4.9) and swiss chard (EC_e = 7.0) (Grieve et al., 2012). Despite the sensitivity of strawberry to salinity, some cultivars are shown to be more tolerant than others. For example, among five strawberry cultivars field-tested under saline irrigation water (EC_{iw} = 2.5 dS m^{-1}) for 240 days, 'Albion' had survival rate of 94% (Ferreira et al., 2019). In another study, 'Ventana' yield was unaffected up to EC_{iw} = 1.7 dS m^{-1}, but reduced drastically beyond that point (Suarez and Grieve, 2013). A recent study evaluating fruit yield, size, brix and plant survival concluded that newer commercial strawberry releases have enough salt-tolerance variability that can be explored for breeding (Ferreira et al., 2019). As southern and central California strawberry farms are located in the coastal valleys, irrigation water quality in these areas has become a major concern for strawberry farmers due to the increased salt concentration. Urbanization and increased groundwater extraction have led to a rise in the salt concentration of groundwaters, while the continuous drought in California has resulted in the depletion of surface waters and increased groundwater pumping. Increased pumping in coastal areas eventually leads to sea water intrusion, adversely impacting coastal ground water supplies. Salinization of the irrigation

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water results in increased salt concentration in the root zone and decreased yield of salt-sensitive crops.

Plants use a large network of genes and develop physiological and biochemical responses for either avoiding or tolerating salt stress (Munns and Tester, 2008). Some common strategies include ion uptake by the roots, ion exclusion from the roots, ion accumulation in vacuoles of root or shoot cells, regulation of ion transport from root to shoot, increased tolerance to high concentrations of toxic ions and accumulation of compatible solutes (Gupta and Huang, 2014; Munns and Tester, 2008; Sandhu and Kaundal, 2018; Sandhu et al., 2018). A previous study involving five commercial cultivars of strawberry established that the plants had an efficient mechanism to sequester Na^+ in roots and stems with little to no Na^+ being transported to leaves (Ferreira et al., 2019). However, specific toxicity of Cl^- played an important role in decreased performance in all strawberry cultivars tested previously in our laboratory (Ferreira et al., 2019; Suarez and Grieve, 2013) and is credited with the destruction of hydathodes, impairing the extrusion of excess Cl^- by leaves and leading to typical symptoms of salt toxicity on the edge of mature leaves (Ferreira et al., 2019). Even though strawberries are sensitive to salinity, different strawberry cultivars display different levels of tolerance (Ferreira et al., 2019; Suarez and Grieve, 2013). Although, variation for salt tolerance is available in the strawberry germplasm, the link between this variation and the genetic mechanisms of salt tolerance is essentially missing. One of the main reasons for this disconnect is the complex genomic organization of the genus *Fragaria* that has many levels of ploidy within wild species (diploid, tetraploid, hexaploid and octoploid). Commercial strawberry (*F.* x *ananassa*) cultivars are octoploid, (2n = 8x = 56) containing four subgenomes (Shulaev et al., 2011). On the other hand, *F. vesca* L. (woodland strawberry) is a diploid (2n = 2x = 14) and is an ideal model system, not only for the cultivated strawberries but also for other members of Rosaceae. The *F. vesca* genome has been sequenced (Shulaev et al., 2011) and a variety of forward and reverse genetic tools have been developed for the functional characterization of strawberry genes. In this study, we evaluated salinity responses of eight *F. vesca* genotypes by comparing physiological parameters in response to salinity and quantifying the expression of different genes known to be involved in the salt-tolerance mechanism.

2. Material and methods

2.1. Experimental setup

Eight *F. vesca* genotypes (PI 551508, PI 551826, PI 551834, PI 552283, PI 552288, PI 602923, PI 616872 and PI 641093) were evaluated in the greenhouse lysimeters at the US Salinity Laboratory (USDA), Riverside, CA (33.973265 latitude, -117.321158 longitude). Each lysimeter sand tank measured 120 cm (L) x 60 cm (W) x 50 cm (D). *F. vesca* seeds were obtained from the USDA GRIN database. These eight genotypes were assigned Genotype numbers (G1 through G8) for convenience {PI 551508 (G1), PI 551826 (G2), PI 551834 (G3), PI 552283 (G4), PI 552288 (G5), PI 602923 (G6), PI 616872 (G7) and PI 641093 (G8)}. The genotypes were asexually propagated as clones to eliminate genetic variability when testing the effect of treatments. Cloned plants were allowed to establish for 4 weeks before being submitted to the salt treatment. During this time, plants were irrigated with water containing basic macronutrients (Control, Table 1). In addition, micronutrients were added in the following composition: Fe 50 μmol L^{-1} added as Fe-DTPA (Sprint 330®), $ZnSO_4.7H_2O$ 1.2 μmol L^{-1}, $CuSO_4.5H_2O$ 0.3 μmol L^{-1}, $(NH_4)6Mo7O_{24}.4H_2O$ 0.1 μmol L^{-1}, H_3BO_3 23 μmol L^{-1}, and $MnSO_4$ 15 μmol L^{-1}. Nutrient solutions were prepared in 890 L reservoirs and pumped to the growing tanks through PVC pipes. Excess water from the cultivation sand tanks drained back to the reservoirs. Roots were exposed to a constant and uniform root-zone salinity. Reservoirs were replenished regularly to account for water lost during evapotranspiration. Plants were irrigated twice daily.

Table 1
Composition of irritation water.

Treatment	EC_{iw} (dS m^{-1})	Ion concentration (mmol$_c$ L^{-1})							
		Cl^-	$SO4^{2-}$	NO^{3-}	$PO4^{3-}$	Na^+	K^+	Ca^{2+}	Mg^{2+}
Control	1.46	1.55	1.65	5.39	1.75	1.88	5	3.4	2.15
Saline	7.94	54.75	11.65	5.39	1.75	46.38	5	14.5	9.75

The experiment was conducted in a randomized complete block design with two irrigation water treatments (control and saline), each replicated thrice. Two cloned plants per genotype were cultivated for each treatment and each replicate.

For the treatment application, the salinity level of irrigation water (EC_{iw}) was gradually increased in three steps, taking three days until the target salinity of 8 dS m^{-1} was achieved by increasing amounts of NaCl, $MgSO_4$, Na_2SO_4, $CaCl_2$ and KCl (Table 1). The level of salinity (8 dS m^{-1}) was chosen based on our preliminary field experiment with *F. vesca* (data not shown). The ratio between cations (Ca = 1.25 Mg = 0.25Na) was maintained and Cl^- was kept as the predominant anion. Macro- and micro-nutrients were retained at the same level in control and saline treatments and the salinity levels of irrigation water were maintained thereafter until harvest.

2.2. Biomass and ion composition

Plants were harvested four weeks after salt treatment and samples were separated into roots, petioles and leaves. Samples were washed with distilled water and dried using paper towels. Fresh weight was recorded immediately and dry weight (dw) was recorded after drying at 70 °C for 96 h. The salt tolerance (ST) index was determined by calculating the ratio between the average biomass of plants under salinity stress and average biomass of control plants.

Dried samples were ground and used for ion analysis. Tissue concentrations of Na, K, Mg, Ca, total-S, Fe, Cu, Mn, Zn and Mo were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Optima 8000) analysis of ground tissues digested with nitric acid digested tissues (PerkinElmer Inc., Waltham, MA). Chloride concentrations were determined by amperometric titration of the nitric-acid extracts (Liu, 1998).

2.3. Chlorophyll concentration and gas exchange measurements

Leaf gas exchange measurements including net photosynthesis (*Pn*), stomatal conductance (*gs*), and transpiration rate (*Tr*) were recorded in both control and salt-treated plants using a Li-Cor 6400 Photosynthesis System (Li-Cor, Lincoln, NE, USA) on the 34th day from the initiation of the salt treatment. The middle leaflet of the most recently fully expanded trifoliate was measured. Readings were taken for six mid leaves per treatment per genotype (one trifoliate per plant). The measurement conditions were set as photosynthetic photon flux density, 1000 $\mu mol_{photon}\, m^{-2}\, s^{-1}$ provided by a red LED light diode source emitting at 670 nm mounted on the top of leaf chamber; operational or chamber ambient CO_2 concentration of 400 $\mu mol_{CO2}\ mol_{air}^{-1}$, and with leaf temperature ranging from 23 to 29 °C. The leaf to air vapor pressure deficit ranged from 1.0 to 2.7 kPa. The photosynthesis system also estimated leaf intercellular CO_2 concentration (*Ci*) according to the leaf gas exchange values. Leaf water-use efficiency (*WUE*) was calculated using the formula that considers the ratio between photosynthetic efficiency (*Pn*) and transpiration (Tr) (WUE = *Pn*/Tr). Leaf chlorophyll concentration of each middle leaflet used for the leaf gas exchange parameter was measured as Soil-Plant Analyses Development (SPAD) reading using a Chlorophyll Meter (SPAD-502, Minolta, Osaka, Japan).

Significant differences were determined at $P \leq 0.05$ among the 8 genotypes at each salt treatment and between the control and salt

treatment for each genotype using the Tukey multi-comparison method in GLM procedure and the *t*-test procedure both in SAS (version 9.3; SAS Institute, Cary, N.C.), respectively.

2.4. Quantitative reverse Transcription-PCR (qRT-PCR) analyses

Genes for qRT-PCR analysis were selected due to their involvement in Na^+ or Cl^- transport during salt stress based on the functional homology with genes characterized in Arabidopsis. Arabidopsis gene sequences were used in Basic Local Alignment Search Tool (BLAST) analysis against the *F. vesca* genome sequence. The *F. vesca* genes with highest homology with Arabidopsis genes were used to design qRT-PCR primers such that at least one primer out of each pair was designed from two exons flanking an intron.

Tissue samples from roots and leaves were taken 48 h after maximum salt concentration (8 dS m^{-1}) was applied as irrigation water. Samples were frozen immediately in liquid nitrogen and RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). DNA contamination was removed treating RNA samples with DNase I, following manufacturer's recommendations (Thermo Scientific, Waltham, MA, USA). The qRT-PCR analysis was performed in a BioRad CFX96 machine using iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad Laboratories, Hercules, CA, USA). For PCR reactions, total volume of 10 µl was used, which contained 1 ng total RNA, 0.75 µM of each of the primers, 0.125 µl iScript™ Reverse Transcriptase and 5 µl of 2x one-step SYBR® Green Reaction mix. The thermocycler conditions were as follows: 50 °C for 10 min, 95 °C for 1 min, then 40 cycles of 95 °C denaturation for 10 s, 57 °C annealing for 30 s, and 68 °C extension for 30 s. For normalization of the expression analysis, *F. vesca Rib 413* (18S rRNA) and *EF1α* genes were used as reference genes. The relative expression differences were calculated by comparing the cycle threshold values of each gene to the reference gene using the following formula:

Normalized $Expression_{sample(GOI)} = \{RQ_{sample\ (GOI)}\} / \{RQ_{sample\ (Ref\ 1)} \times RQ_{sample\ (Ref\ 2)} \times \ldots RQ_{sample\ (Ref\ n)}\}^{1/n}$. Where: RQ is relative quantity of a sample, Ref is reference target in a run that includes one or more reference targets in each sample and GOI is gene of interest. The amplification specificity was tested by melt curve analysis by ramping the temperature to 95 °C for 10 s and back to 65 °C for 5 s followed by increments of 0.5 °C/cycle up to 95 °C.

3. Results

3.1. Effect of salinity on root and shoot biomass of the strawberry genotypes

Evaluation of shoot and root biomass of eight genotypes showed that the salinity response was genotype dependent (Fig. 1). G8 was the best performer under salinity for both shoot and root biomass with an average shoot dry weight of 7.10 g and an average root dry weight of 1.84 g (Fig. 1, Supplemental Fig. 1). G3 and G4 were the lowest shoot and root biomass producers, respectively. G8 was the most vigorous genotype both for root and shoot biomass under the control and salinity treatments (Fig. 1, Supplemental Fig. 1).

Salt tolerance (ST) index (the ratio between biomass accumulation under salinity stress and under controlled condition) for shoot ranged from 0.41 to 0.86 (Fig. 1A). Salinity led to a reduction in root and shoot biomass in most genotypes. The highest reduction in shoot biomass (59%) was observed for G5 under salinity conditions, compared with the control (Fig. 1A). On the other hand, G1 displayed the lowest biomass reduction of 14% under salinity. For root biomass, G8 performed the worst, with 47% reduction under salinity, while G1 performed the best with only 14% reduction (Fig. 1B). The most salt-tolerant clone, G1, was among the top yielders for root and shoot biomass. G4, which was a genotype with low ST index was the lowest in root biomass yield under salinity.

3.2. Effect of salinity on ion composition

All the genotypes had increased Na concentrations in roots, petioles and leaves, however, the extent of the increase varied with genotype (Fig. 2A). The concentration of Na was the highest in roots, followed by petioles, with the least concentration found in leaves of plants under both control and salinity treatments. The average increase in Na concentration under salinity was 1.98, 3.78, and 3.82-fold in roots, petioles and leaves, respectively (Fig. 2A). When compared with the control, plants under salinity increased their Na concentration ranging from 1.67 to 2.27-fold in roots, 2.64–4.96-fold in petiole and 2.21–5.65-fold in leaves. In plants irrigated with high-salinity water, Na concentration varied from 218.65–284.41 mmol kg^{-1} dw in roots, 87.46 to 161.21 mmol kg^{-1} dw in petioles and 25.59 to 62.92 mmol kg^{-1} dw in leaves (Fig. 2A). G6 and G8 plants had high tissue concentrations of Na under the salinity treatment, in roots, petioles and leaves. G1, which had high ST index, displayed low Na concentration in all three tissues and G7 had high Na concentration in roots but low concentration in petiole and leaves (Fig. 2A).

The concentration of Cl also increased in different tissues of plants under salinity (Fig. 2B). Compared to plants under the control treatment, the average increase in tissue Cl concentration was 3.21, 5.78 and 4.09-fold in roots, petioles and leaves, respectively, in plants under the salinity treatment. The average Cl concentration in plants under the control treatment was the highest in leaves, followed by roots and petioles. However, under salt treatment, leaves had the highest concentration of Cl followed by petioles and roots (Fig. 2B). For plants irrigated with high-salinity water, Cl concentrations varied from 317.96 to 443.46 mmol kg^{-1} dw in roots, 402.79 to 596.17 mmol kg^{-1} dw in petioles and 369.95 to 678.74 mmol kg^{-1} dw in leaves. Of the eight genotypes tested, G1 stored the least amount of Cl in roots, petioles or leaves under salinity (Fig. 2B). Under salinity treatment, G4 the second to lowest in shoot biomass and the lowest in root biomass, displayed the highest increase in Cl accumulation in petioles (7.15-fold increase when compared with control) and leaves (5.61-fold increase compared with the control). Interestingly, G4 had the least increase in Cl concentration in roots (Fig. 2B).

For plants under the salinity treatment, the average Cl tissue concentration was 385.15, 490.79 and 522.01 mmol kg^{-1} dw in roots, petioles and leaves, respectively. On the other hand, the average Na concentration in the salinity treatment was 242.64, 128.82 and 44.14 mmol kg^{-1} dw in roots, petioles and leaves, respectively. The concentration of Na accumulated in roots, petioles and leaves was significantly lower than that of Cl ($P \leq 0.05$).

Tissue K concentration varied considerably in different tissues of strawberry plants, with petioles containing the highest amount, followed by leaves, then roots. For plants under the salt treatment, the average tissue K concentration per plant decreased by 22.8% compared with the control (Fig. 2C). There was 27.3%, 22.5% and 18.5% decrease in K concentration in roots, petioles and leaves, respectively. On average, and in all tissues, G1 displayed the lowest, and non-significant, decrease in K concentration (14.6%) under salinity when compared with the control, while G6 had the highest decrease (28.1%). Under salt treatment, the highest K concentration in leaves was found in G7 (Fig. 2C).

3.3. Effect of salinity on gas exchange and chlorophyll concentration

Comparison of different genotypes revealed that the genotypes G1, G5 and G7 had significant reduction ($P \leq 0.05$) in stomatal conductance (*gs*) and transpiration rate (*Tr*) under salinity when compared with the control (Fig. 3). Leaf intercellular CO_2 concentration (*Ci*) of salt-stressed plants was significantly reduced when compared with the controls in G1 and G7 (Fig. 3). On the other hand, leaf water use efficiency (WUE) of salt stressed plants was significantly ($P \leq 0.05$) higher than that of control plants in G1 and G7 (Fig. 3). The salinity stress

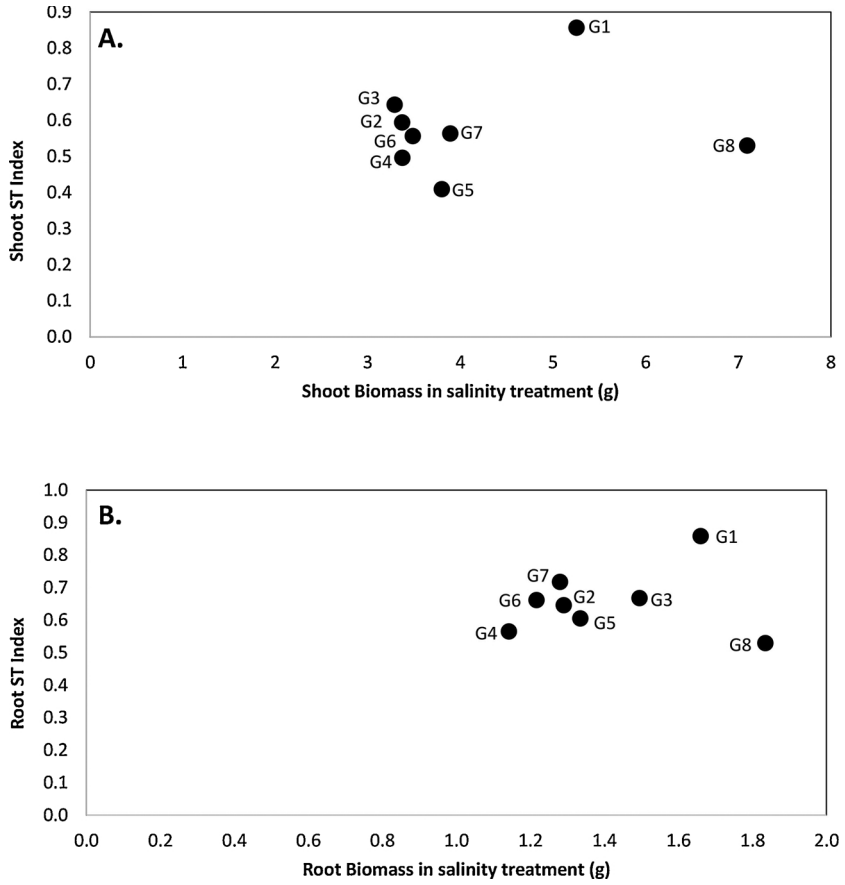

Fig. 1. Relationship between total biomass in salinity treatment and salt tolerance. A) Relationship between shoot salt tolerance (ST) index and shoot biomass. B) Relationship between root salt tolerance (ST) index and shoot biomass.

applied (EC_{iw} = 8.0 dS m^{-1}) appeared not to affect photosynthetic rate (*Pn*) of the eight tested strawberry genotypes. Overall, there was no significant difference ($P > 0.05$) in *Pn* between control and salt-stressed plants for any of the genotypes (Fig. 3). The leaf chlorophyll SPAD readings were not affected by salt stress in most genotypes (Fig. 3). Yet, the SPAD readings of G7 of salt stressed plants were significantly ($P \leq 0.05$) higher than that of control plants.

3.4. Expression analyses

A set of 11 genes known to play important roles in salt stress response was used to evaluate plant responses to salinity through their expression in plants irrigated with waters of low (control, EC_{iw} = 1.4 dS m^{-1}) and elevated (EC_{iw} = 8.0 dS m^{-1}) salinity (Supplemental Table 1). These genes were selected based on functional conservation with the genes studied in model plants. These genes are classified into two groups based on the importance of the ions involved in salt toxicity as follows:

3.4.1. Genes involved in Na^+ transport

We studied the expression of 7 genes (*SOS1, SOS2, SOS3, NHX1, NHX2, AKT1, SAL1*) that are known to be critical for Na^+ transport. Only one genotype, G8, displayed simultaneous significant ($P \leq 0.05$) upregulation of the *SOS1* gene in roots and leaves, while some other genotypes exhibited upregulation in roots or leaves, but not in both (Fig. 4). *SOS1* was downregulated in roots of three genotypes (G4, G6 and G7) under salinity. Four genotypes (G1, G2, G3 and G8) had significant ($P \leq 0.05$) upregulation of the *SOS2* gene in roots under salinity, compared with the control. Also, only G3 and G6 displayed significant upregulation of *SOS2* in leaves when compared with the control (Fig. 4). Interestingly, *SOS3* and *AKT1* showed similar expression patterns in roots and leaves of all eight genotypes. Neither genes were upregulated in roots of plants under salinity in any of the genotypes, but were significantly upregulated in G5 and G6 leaves when compared with control (Fig. 4). In addition to G5 and G6, *SAL1* was also upregulated in leaves of G7 and G8. Interestingly, G1, the best performer with the highest salt tolerance index, exhibited significant down regulation for *SOS3*, *AKT1* and *SAL1* in roots (Fig. 4).

For the genes involved in sequestering Na^+ into vacuoles, *NHX1* was significantly upregulated in roots of G1, G2 and G8 and in leaves of G1, G2, G3 and G8. For the two worst performers for the ST index, G4 and G5, *NHX1* and *NHX2* were significantly downregulated in roots, respectively (Fig. 4). *NHX2* was upregulated under salinity in leaves of G1 and in roots of G2, G3 and G8 when compared with the control.

3.4.2. Genes involved in Cl^- transport

Four genes (*CLC_G, CLC_C, SLAH3* and *ALMT12*) known to play important roles in Cl^- transport were characterized under both control and salinity conditions in all eight genotypes (Fig. 5). CLC_G was induced in G8, whereas CLC_C was induced in G5 under salinity in roots. SLAH3 was upregulated in roots of G2 and G6 and was downregulated in G4 and G7 (Fig. 5). ALMT12 was upregulated in G2 roots, but was downregulated in G6 (Fig. 5). *ALMT12* was upregulated in five genotypes (G1, G2, G4, G5, and G6) out of eight. SLAH3 was upregulated in G5, G6 and G8. Two genes, *CLC_C* and *ALMT12*, were significantly upregulated in G1 leaves under salinity. *CLC_C* was significantly downregulated in leaves of G5, G7 and G8 (Fig. 5).

4. Discussion

In order to understand the roles of various biochemical, physiological and genetic players during salt stress, we studied eight woodland

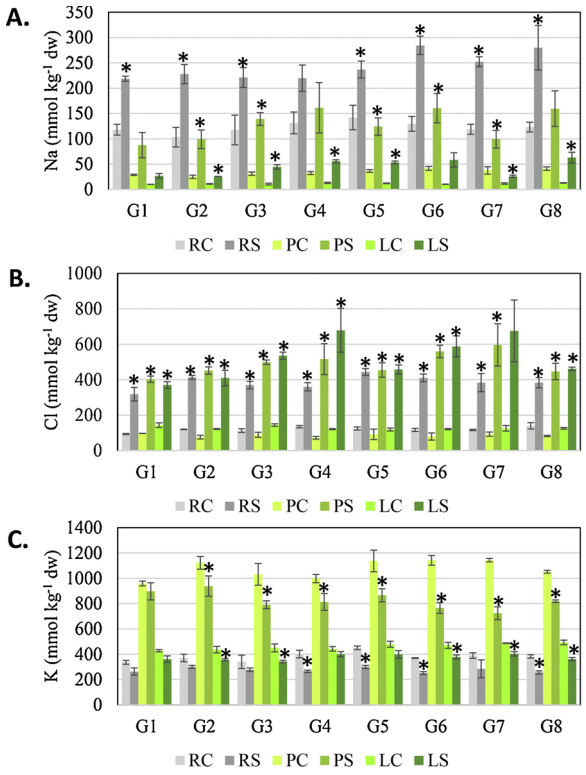

Fig. 2. Root, petiole and leaf ion concentrations of 8 woodland strawberry genotypes in the control and the salt treatments. A) Average Na concentration. B) Average Cl concentration. C) Average K concentration. RC, PC and LC are root, petiole and leaf control, respectively; RS, PS and LS are root, petiole and leaf salt treatment, respectively. Asterisk indicates significant ($P \leq 0.05$) difference between the control and the salt treatment. Error bars represent standard errors.

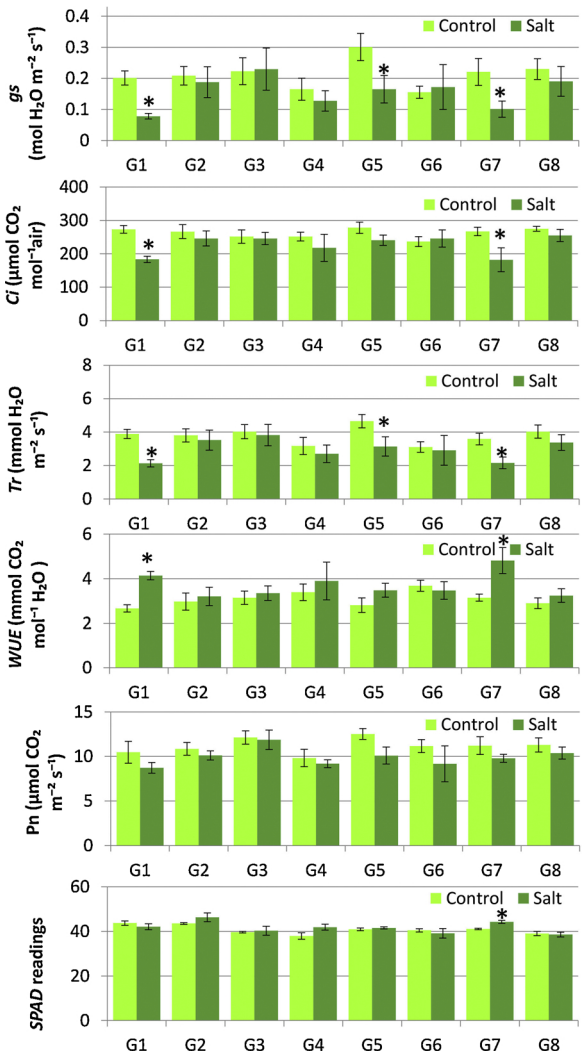

Fig. 3. Leaf gas exchange of stomatal conductance (*gs*), transpiration rate (*Tr*), intercellular CO_2 concentration (*Ci*), water use efficiency (*WUE*), net photosynthetic rate (*Pn*), and leaf SPAD chlorophyll reading of 8 strawberry genotypes irrigated with two waters {Electrical Conductivity (EC) = 1.46 dS m^{-1} as a control and EC = 8.0 dS m^{-1} as a saline treatment}. Data are presented as means with a sample size of n = 6 (leaf, one leaf per plant). Asterisk indicates the significant difference ($P \leq 0.05$) between the two salinities within a genotype under 34 days of salt stress. Different lowercase letter marks a significant difference among the 8 genotypes.

strawberry genotypes. To eliminate genetic variability within a genotype, all the plants for a genotype were cloned from a single plant. All eight genotypes displayed reduction in shoot and root dry weight under the salt stress applied as irrigation water (EC_{iw} = 8.0 dS m^{-1}) using a lysimeter system that maintains constant root-zone salinity. However, the rate of biomass reduction among different genotypes varied tremendously. G1 had maximum ST index with minimum biomass reduction (14%), while G5 displayed maximum reduction (59%) when compared with plants under the low-salinity control treatment (Fig. 1).

We observed that Na concentration was the highest in roots, followed by petioles, then leaves in plant under both control and salt treatments (Fig. 2A). For most other plant species, concentration of Na is normally lower in roots when compared with shoots (Sandhu et al., 2018). In strawberry, a high concentration of Na in roots and petioles, while leaves maintain low concentrations of Na, suggests the existence of a specialized mechanism that holds Na in petioles and roots, restricting its accumulation in leaves. A similar lack of increase in leaf Na was also reported for five commercial (polyploid) cultivars of strawberry irrigated with waters ranging from 0.7 to 2.5 dS m^{-1} (Ferreira et al., 2019). The genotypes with low ST index such as G6 and G8, had high tissue concentrations of Na when plants were irrigated with saline water of 8 dS m^{-1}, while the most salt-tolerant genotype, G1, displayed low Na concentration in both leaves and roots (Fig. 2A). These observations suggest that either G1 takes little Na^+ into roots or that there is an efficient Na^+ exclusion mechanism present in roots. On the other hand, G8 showed maximum increase in Na concentration in roots of plants under the salinity treatment, compared with control plants. The increase in leaf Na for G8 was 4.8-fold compared with the control (Fig. 2A). G8 was the best biomass producer under control but had a low ST index (0.53), reflecting its poor biomass production under salinity. Thus, the low ST index for G8 may be due to its inability to exclude Na^+ from roots and leaves. The fact that plant in this experiment showed higher leaf accumulation of Na than the five commercial strawberries previously studied (Ferreira et al., 2019) may be due to the fact that we have submitted our plants to 3 times more NaCl than the plants in that field experiment (EC_{iw} = 2.5 dS m^{-1}). This may also indicate that the control mechanism that prevents Na translocation to leaves may have a salinity concentration threshold. Thus, although Ferreira et al. (2019) have concluded that Cl was the most toxic ion to commercial strawberries irrigated with water of EC_{iw} = 2.5 dS m^{-1}, Na may also play a role in reducing plant biomass when its concentration is high in irrigation water.

The Cl concentration in plants under salt treatment was the highest in leaves followed by petioles and roots, suggesting that Cl is not well regulated by roots (Fig. 2B). Some herbaceous plants, such as Jerusalem artichoke also had the ability to maintain Na in roots and tubers under treatments with EC_{iw} up to 12 dS m^{-1}, while no Na was transferred to stems and leaves. In contrast, Cl increased in all tissues of Jerusalem

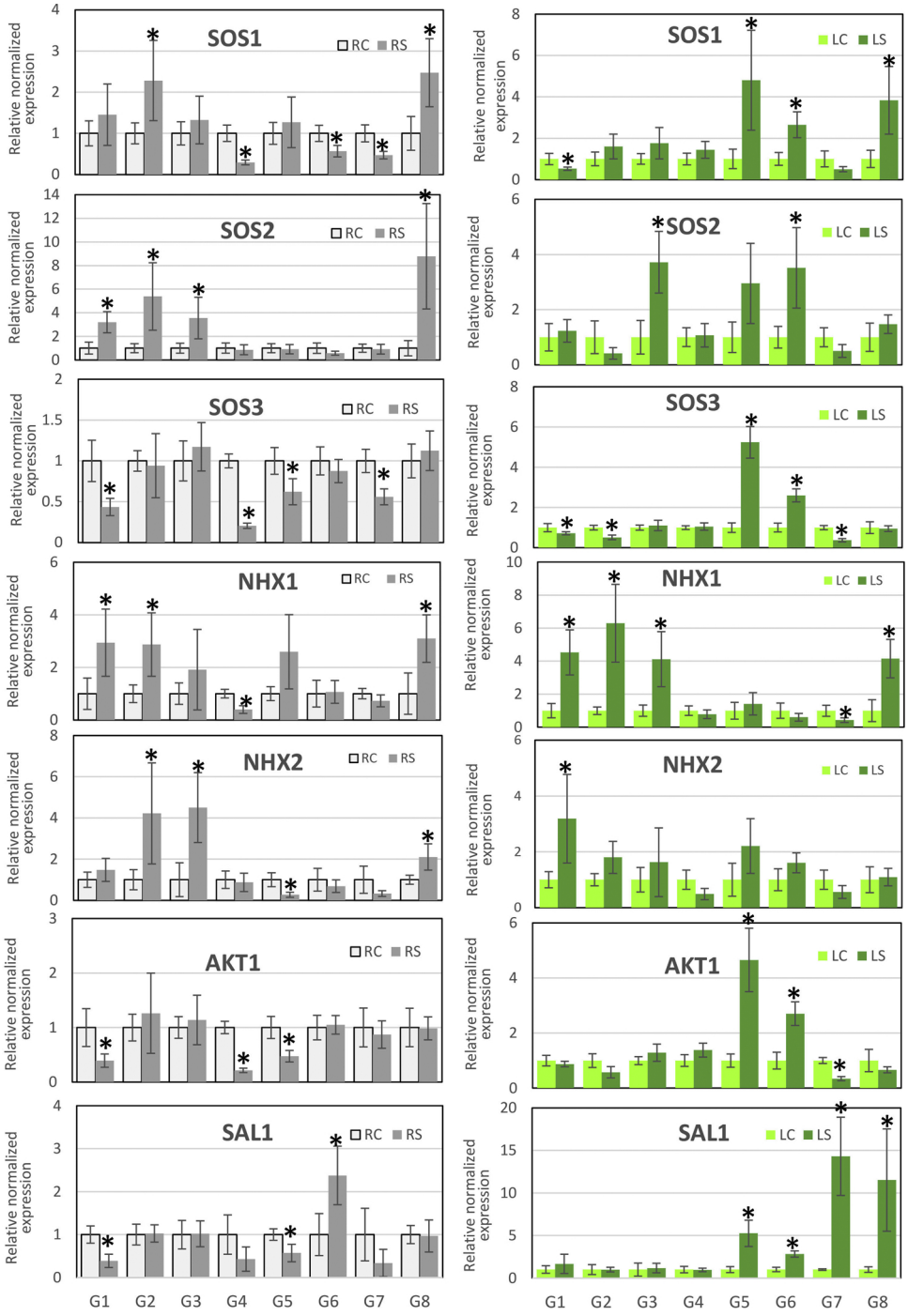

Fig. 4. Expression of strawberry genes involved in Na^+ transport in the control and salt treatments. Asterisk indicates significant ($P \leq 0.05$) difference between the control and the salt treatment. Error bars represent standard error. LC and RC are root and leaf control, respectively; LS and RS are root and leaf salt treatment, respectively.

artichoke with increasing salinity (Dias et al., 2016). Among eight strawberry genotypes, G1 stored the least amount of Cl in all three tissues analyzed, suggesting that this genotype displays the best control of Cl^- absorption from soil to plant tissue (Fig. 2B). On the other hand, G4 had the highest (5.6-fold) increase in Cl accumulation in leaves and the lowest (2.7-fold) increase in Cl accumulation in roots implying a reduced ability of this genotype to hold Cl^- in roots in comparison to others, thus explaining its poor performance under salinity.

In plants under salinity treatment, the average Cl concentration in leaves (522.01 mmol kg^{-1} dw) was > 11-fold higher than the average Na concentration (44.14 mmol kg^{-1} dw). This indicates that Cl^- toxicity may be more critical than Na^+ in strawberry. Previous studies on commercial strawberry cultivars also determined that all five commercial strawberries evaluated had an efficient mechanism to sequester Na^+ primarily in roots and stems with little to no Na^+ being transported to leaves; and that Cl^- played an important role in decreased yield performance and survival of both greenhouse and field-tested plants (Ferreira et al., 2019; Khayyat et al., 2007; Suarez and Grieve, 2013). The commercial strawberry variety, 'Ventana', did not show yield loss in mixed cation-sulfate ion composition but displayed severe yield loss when irrigated with $CaCl_2$-dominated water, indicating that Cl^- has specific ion toxicity in strawberry (Suarez and Grieve, 2009,

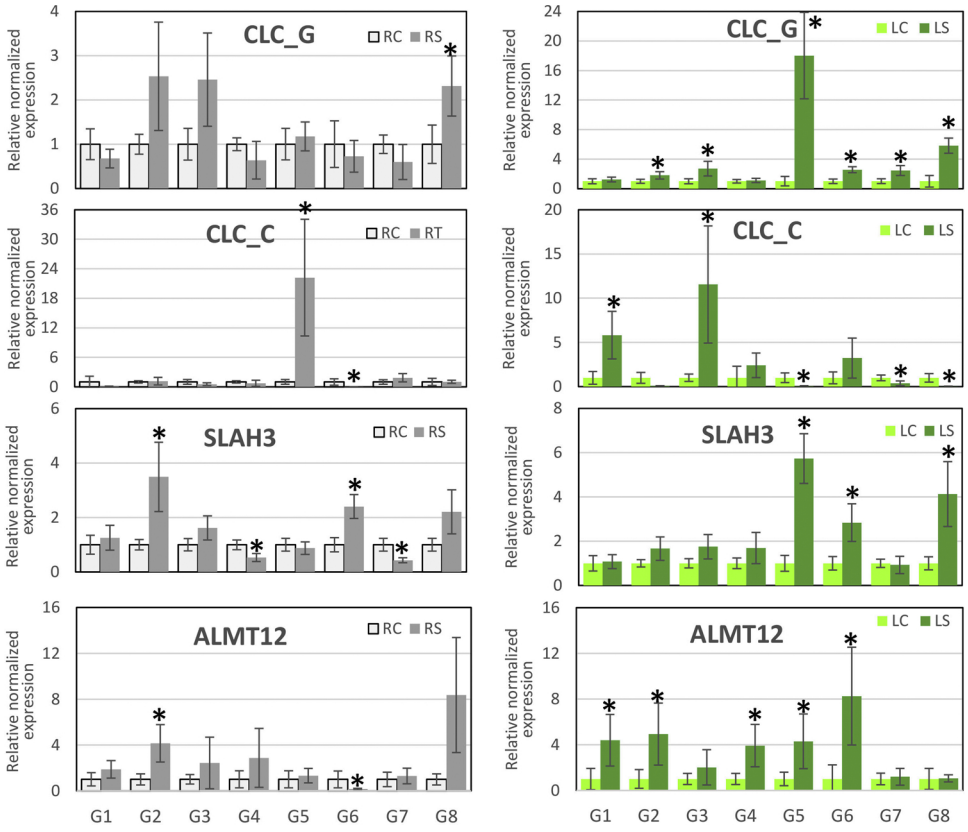

Fig. 5. Expression of strawberry genes involved in Cl^- transport in the control and salt treatments. Asterisk indicates significant ($P \leq 0.05$) difference between the control and the salt treatment. Error bars represent standard error. LC and RC are root and leaf control, respectively; LS and RS are root and leaf salt treatment, respectively.

2013). Correlation between ion concentrations in different tissues during salinity revealed that only root Cl concentration had a high significant correlation (r = -0.79, P = 0.02) with shoot ST index (Supplemental Table 2). Higher Cl concentrations in irrigation water reduces photosynthesis and carbohydrate production, which affects fruit yield and quality (Martinez Barroso and Alvarez, 1997).

The K/Na ratio is commonly used as an indicator of salt tolerance in plants. During salt stress, tissue Na concentration goes up and tissue K concentration goes down. Similarly, in diploid strawberry, Na and K concentrations in different tissues showed an inverse relationship (Fig. 2). However, this inverse relationship was not found in a previous work with five commercial cultivars in that Na concentrations increased in both roots and petioles with salinity, but K concentration in tissues of most commercial cultivars tested remained stable, except for 'Monterey' (Ferreira et al., 2019). The decreased K concentration found in our plants irrigated with EC_{iw} = 8 dS m^{-1} may be explained by the higher concentration of NaCl used in this study compared to the previous study (EC_{iw} = 2.5 dS m^{-1}). In this work, G1, G2 and G7 were the three best genotypes for high K/Na ratio in petioles and leaves under salinity treatment (Supplemental Table 3), which may partially explain the high shoot ST index for these genotypes. However, the correlations between the shoot ST index and K/Na ratios in roots, petioles and leaves under salinity were 0.12, 0.61, and 0.48, respectively (Supplemental Table 2). Low non-significant correlations between shoot ST index and K/Na ratios in different tissues indicate that K/Na ratio presents only one piece of the mechanism and may not be used as a single determining factor to evaluate salt tolerance in strawberry.

For *Pn,* there were no significant differences found among the eight genotypes or between the two treatments. However, G5 and G6 had a reduction in their *Pn* caused by the salt stress (Fig. 3). It has been reported previously that *Pn* of strawberry plants under ambient CO_2 concentrations was not affected by either 30 mM or 60 mM NaCl treatment for the cultivar 'Elsanta', but showed reduction for 'Korona' plants under 60 mM NaCl treatment (Saied et al., 2003). It appears that *Pn* of different strawberry genotypes or cultivars responds to salt stress differently. Interestingly, G1 had significant reduction in *gs, Ci, Tr*, which in turn led to a significant increase in *WUE* under salt treatment, compared with control (Fig. 3). The observed lower *Ci* and *Tr* values can be explained by the lower *gs* observed, as *gs* directly impacts *Tr* and reduces CO_2 supply to leaves resulting in lower *Ci* and higher *WUE* (Fig. 3). G1 was the top performer based on its ST index, fact that can be explained by the combination of its improved *WUE* with its lower Na and Cl tissue accumulation under salinity. These factors combined may have had a direct effect on G1's relative performance under salinity.

Stomatal opening/closing is critical for plant water uptake and in maintaining CO_2 concentration for photosynthesis. Aquaporins, small integral membrane proteins, facilitate water transport across biological membranes (Maurel et al., 2015). During initial stages of salinity stress, expression of aquaporin genes drops, probably to reduce water loss; however, at later stages the expression is upregulated to maintain water homeostasis (Pawłowicz and Masajada, 2019; Sade et al., 2010). Although, aquaporins are considered vital during salinity stress, the conflicting roles of different aquaporin members in salt tolerance are not fully understood yet.

Leaf guard cells control opening and closing of stomata by regulating turgor pressure. High turgor pressure in guard cells leads to high *gs*. The guard cell turgor pressure is controlled by a series of biochemical and physiological processes that are affected by the environment, time and cell water-related factors. Under drought or salt stress, ABA signaling pathway plays a key role in stomatal regulation with excess ABA leading to stomatal closure (Roelfsema and Hedrich, 2005; Sah et al., 2016; Sreenivasulu et al., 2012). Increased accumulation of salt in plant tissues during salt stress triggers ABA signaling cascade of a series of genes for an ABA-induced stomatal closure (Roelfsema and Hedrich, 2005; Sah et al., 2016; Shaterian et al., 2005). However, further experiments are needed to provide direct evidence on ABA regulation of stomatal closure under different salt-stress durations in strawberry plants.

Several different pathways are involved in regulating salt tolerance in plants. In many plants, Na^+ is known to be an important ion that regulates ion toxicity during salt stress (Liu et al., 2015; Munns and Tester, 2008; Sandhu et al., 2017; Sandhu and Kaundal, 2018; Sandhu et al., 2018). However, for a sensitive crop such as strawberry, Cl^- toxicity is shown to be extremely critical (Ferreira et al., 2019; Suarez and Grieve, 2013). In this investigation, we evaluated the association between different genes involved in Na^+ and Cl^- transport with salt tolerance in eight genotypes of the diploid woodland strawberry *F. vesca*. Thus, we conducted expression analysis of 7 genes (*SOS1, SOS2, SOS3, NHX1, NHX2, AKT1, SAL1*) involved in Na^+ transport and 4 genes (*CLC_G, CLC_C, SLAH3, ALMT12*) involved in Cl^- transport.

One of the important pathway that regulates Na^+ transport is the salt overly sensitive (SOS) pathway (Qiu et al., 2002). SOS pathway controls exclusion of Na^+ from plant roots via three genes, *SOS1, SOS2* and *SOS3*. Upon sensing salt stress, the plant sends a Ca^{2+} signal that activates SOS3, which interacts with SOS2 to trigger its kinase activity, which in turn leads to phosphorylation of SOS1 (Gupta and Huang, 2014; Shi et al., 2002). SOS1, a plasma membrane associated protein, when phosphorylated, regulates Na^+ efflux from the root (Shi et al., 2002). Manipulations of SOS proteins have provided enhanced tolerance to salt in heterologous species (Liu et al., 2015; Shi et al., 2003; Zhang and Blumwald, 2001). Of the three genes involved in Na^+ exclusion, *SOS2* was significantly upregulated in the genotype (G1), the genotype with the highest ST index (Fig. 4). *SOS1* displayed similar expression between control and the salt treatment and *SOS3* was down-regulated in salt treatment. These observations suggest that the components of Na^+ exclusion are not very active during salt stress in strawberry. On the other hand, *SOS2*, in additions to *NHX1* and *NHX2*, is also known to play an important role in the sequestration of Na^+ into plant vacuoles (Batelli et al., 2007). Two lines with high ST index (G1 and G2) exhibited upregulation of *NHX1* in roots and leaves of plants under salt treatment (Fig. 4). *NHX2* was also upregulated in leaves of G1 and roots of G2 under salinity, suggesting that sequestration of Na^+ into vacuoles may be an important component trait for the salt tolerance mechanism in strawberry. High concentration of Na^+ in roots, when compared with petioles and leaves, also indicates sequestration of Na^+ concentration in root vacuoles in strawberry plants (Fig. 2).

Recent literature suggests that Cl^- toxicity has a major impact in several plant species during salt stress (Ferreira et al., 2019; Sandhu and Kaundal, 2018; Suarez and Grieve, 2013). Chloride channels, (CLCs), slow-type anion channel associated homolog (SLAHs) and aluminum-activated malate transporters (ALMTs) are some of the important players that have been identified to be critical during Cl^- transport in plants (Li et al., 2017). Of the four chloride-transport genes analyzed in our investigation, *CLC_C* was significantly upregulated in leaves of plants under salinity in two genotypes with the highest ST index (G1 and G3), while down-regulated in the genotype with the lowest ST index (G5) (Fig. 5). These observations indicate that chloride channels are important in managing Cl^- stress in strawberry. However, testing and characterization of additional players that are involved in Cl^- transport is warranted in strawberry.

In this investigation we have attempted to tackle the complex problem of salinity by linking the biochemical and physiological responses with the underlying genetic mechanisms in *F. vesca*. After evaluation of biomass and ionic accumulation (mainly Na and Cl), physiological, and genetic parameters of eight diploid woodland strawberries, we can conclude that the identification of genetic mechanisms regulating salt tolerance can be more important in developing commercial strawberry cultivars tolerant to salt than simply basing our screening on K/Na ratio and physiological parameters. Upregulation of genes involved in Na and Cl transport are directly involved in exclusion or accumulation of toxic ions in plant tissues and, eventually, in plant yield and survival under salinity stress.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2019.04.071.

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