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Research article

Stomatal and non-stomatal limitations are responsible in down-regulation of photosynthesis in melon plants grown under the saline condition: Application of carbon isotope discrimination as a reliable proxy

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ABSTRACT

Salinity is one of the most severe environmental stresses limiting agricultural crop production worldwide. Photosynthesis is one of the main biochemical processes getting affected by such stress conditions. Here we investigated the stomatal and non-stomatal factors during photosynthesis in two Iranian melon genotypes "Ghobadlu" and "Suski-e-Sabz", as well as the "Galia" F1 cultivar, with an insight into better understanding the physiological mechanisms involved in the response of melon plants to increasing salinity. After plants were established in the greenhouse, they were supplied with nutrient solutions containing three salinity levels (0, 50, or 100 mM NaCl) for 15 and 30 days. With increasing salinity, almost all of the measured traits (e.g. stomatal conductance, transpiration rate, internal to ambient CO_2 concentration ratio (C_i/C_a), Rubisco and nitrate reductase activity, carbon isotope discrimination ($\Delta^{13}C$), chlorophyll content, relative water content (RWC), etc.) significantly decreased after 15 and 30 days of treatments. In contrast, the overall mean of water use efficiency (intrinsic and instantaneous WUE), leaf abscisic acid (ABA) and flavonol contents, as well as osmotic potential (Ψ_S), all increased remarkably with increasing stress, across all genotypes. In addition, notable correlations were found between $\Delta^{13}C$ and leaf gas exchange parameters as well as most of the measured traits (e.g. leaf area, biomass, RWC, Ψ_S, etc.), encouraging the possibility of using $\Delta^{13}C$ as an important proxy for indirect selection of melon genotypes with higher photosynthetic capacity and higher salinity tolerance. The overall results suggest that both stomatal and non-stomatal limitations play an important role in reduced photosynthesis rate in melon genotypes studied under NaCl stress. This conclusion is supported by the concurrently increased resistance to CO_2 diffusion, and lower Rubisco activity under NaCl treatments at the two sampling dates, and this was revealed by the appearance of lower C_i/C_a ratios and lower $\Delta^{13}C$ in the leaves of salt-treated plants.

1. Introduction

Salinity is one of the major environmental stresses affecting the performance of many crop plants, especially in arid and semiarid climates, where population growth, water shortage and land degradation are major concerns (Munns and Tester, 2008; Geissler et al., 2010). Salinization affects 20% of total arable land on Earth and it has been estimated that up to 50% of the irrigated land would be salinized by the year 2050 (Wang et al., 2003; Porcel et al., 2012).

Most plants exposed to salinity stress show lower CO_2 uptake by their leaves than the same plants not subjected to stress (Dadkhah, 2013). The photosynthetic capacity of plants grown under saline conditions is lower depending on the severity and/or duration of the adverse condition, genotypic difference among the examined cultivars as well as plant age (Hester et al., 2001; Arquero et al., 2006; Flexas et al., 2006). Many studies have concluded that this reduction is a

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consequence of several physiological responses including lower stomatal conductance, a decline in specific metabolic processes in carbon assimilation, or a combination of these parameters (Flexas et al., 2004; Zhang et al., 2009). These effects occur due to limited uptake of water and imposed osmotic stress, interference with uptake of essential elements and modification of ion balances, as well as by salt accumulation in the cytoplasm of photosynthetic cells (Moinuddin et al., 2016).

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the main enzyme involved in CO_2 fixation in C_3 plants, and its activity is rapidly changed for adjusting the photosynthetic carbon reduction cycle under stress conditions (Field et al., 1998). Therefore, monitoring of its activity provides information on limitations of carboxylation arising from adverse environmental conditions such as salinity (Flexas et al., 2004).

Since salinity strongly restricts the availability of atmospheric CO_2 through stomatal closure, the absorption of energy by light-absorbing pigments exceeds the rate of its consumption by the Calvin cycle, i.e. the use of NADPH by the dark reactions of photosynthesis is reduced (Foyer and Noctor, 2005). The excess energy may accelerate the photodamage to photosystem II (PSII) complex, the most sensitive component of the photosynthetic apparatus, via the transfer of electrons to oxygen to form superoxide radicals by a process known as the Mehler reaction that initiates further reactions to produce more harmful oxygen radicals (Melis, 1999; He et al., 2009).

The activity of nitrate reductase (NR), as the first enzyme in the nitrate assimilation process (Solomonson and Barber, 1990), has been shown to decrease in saline-stressed leaves of different plant species, including maize (Abd El-Baki et al., 2000), tomato (Debouba et al., 2006, 2007; Hayat et al., 2012), mulberry (Surabhi et al., 2008), sunflower and safflower (Jabeen and Ahmad, 2011) as well as cucumber seedlings (Li et al., 2019), while an increase in NR activity has been observed in bean (Misra and Dwiverdi, 1990) and in annual ryegrass (Sagi et al., 1997) under such stress condition. These inconsistent results emphasized the importance of investigation on the salinity stress effects on the leaf NR activity in a wider number of species including melons.

Salinity alters the nitrogen (N) metabolism in plants through inducing specific ion effects and nutrient imbalances, resulting in N deficiency which generally decreases crop growth and consequently the harvestable yield (Katerji et al., 2009). Optical sensors are considered as non-destructive and promising alternative approach to N determination after destructive harvests. They indirectly measure nitrogen content from indicator compounds that are sensitive to crop nitrogen content (Tremblay et al., 2012; Padilla et al., 2014). Here, we used the optical sensor, DUALEX (Force A, Orsay, France) for the determination of leaf chlorophyll and epidermal flavonol content to assess the nitrogen status in melon plants under salinity stress (Tremblay et al., 2009). The content of leaf chlorophyll is closely linked to plant N content (Schepers et al., 1996). Also, it has been proved that flavonol content increases under lower nitrogen availability (Bragazza and Freeman, 2007; Liu et al., 2010) and usually is inversely related to the chlorophyll content (Padilla et al., 2014). Due to their opposing relationships to crop nitrogen content, the ratio between chlorophyll and flavonol contents, known as NBI, has been suggested as a sensitive index for plant N status (Cartelat et al., 2005; Tremblay et al., 2012).

The plant stress hormone abscisic acid (ABA) – a lipophilic phytohormone – plays an important role in regulating different plant developmental processes as well as the adaptive responses to a wide range of abiotic stresses causing cell dehydration such as drought and salinity (Mahajan and Tuteja, 2005; Tao et al., 2011; Zheng et al., 2019). The increased level of endogenous ABA in response to water or salt stress is a cell signaling process, including initial stress signal recognizing, cellular signal transduction and regulation of expression of genes responsive in ABA biosynthesis (Zhu, 2002). It has been proven that ABA alleviates the adverse effects of NaCl on photosynthesis, growth and assimilate translocation (Popova et al., 1995). This phytohormone is produced in roots and transported to leaves where it binds to receptors at the surface of the plasma membrane of stomatal guard cells (Babu et al., 2012). Therefore elevated ABA helps plants to acclimate to lower water availability by promoting stomatal closure that prevents excessive transpiration (Kumar et al., 2013).

Discrimination against the heavy carbon isotope, ^{13}C, during carbon assimilation into C_3 plant biomass occurs because of differences in diffusion rates of ^{12}C and ^{13}C, and fractionation by enzymatic carboxylation reactions, leading to ^{13}C depletion in plant organic matter compared with atmospheric CO_2 (Farquhar et al., 1982, 1989). Plants, thus, contain a lower ratio of ^{13}C–^{12}C than CO_2 in the ambient air. Carbon isotope discrimination ($\Delta^{13}C$) of C_3 plant leaves is mainly determined by the relative contribution of diffusive and enzymatic discrimination that is related to C_i/C_a, the ratio of CO_2 mole fraction in the leaf intercellular spaces (C_i) to that in the ambient atmosphere (C_a) (Farquhar and Richards, 1984). Therefore, the most general model (Farquhar et al., 1982) describing overall carbon isotope fractionation during photosynthesis in C_3 plants is based on the two main discriminating steps: discrimination during CO_2 diffusion from the ambient air into the leaves through stomata (4.4‰, a) and during its carboxylation by Rubisco, the primary carboxylating enzyme in C_3 plants and phosphoenolpyruvate carboxylase, PEPC (between 27 and 30‰, b) as follows:

$$\Delta = a + (b–a)\ C_i/C_a \quad (1)$$

Changes in environmental conditions affecting stomatal conductance, and therefore C_i/C_a, cause changes in photosynthetic discrimination in C_3 leaves. If leaf C_i reduces with decreasing g_s, the decline in A_n is because of stomatal factors; otherwise, the decrease in A_n will be owing to non-stomatal effects (Farquhar and Sharkey, 1982; Hejnák et al., 2016; Xia et al., 2017). Based on this, carbon isotope signature of plant organic matter (OM), in general, represents the discrimination of carbon isotopes in a given photosynthesizing system during photosynthetic carbon assimilation (Farquhar et al., 1989) and also provides a reliable estimation of C_i/C_a, as a long term indicator of plant metabolism (Shaheen and Hood-Nowotny, 2005).

Plant ability to efficiently modulate the relationship between water consumption and carbon assimilation is a typical approach widely used for assessing the plant adaption to stressors including salinity and limited water supply (Xia et al., 2017). At the leaf level, WUE may be defined as the ratio of net photosynthesis (A_n) to transpiration (E), which is known as instantaneous water use efficiency (Polley, 2002; Esmaeilpour et al., 2016), or as the ratio of the net photosynthetic rate to stomatal conductance (g_s), that is called intrinsic water use efficiency (WUE_i) (Pascual et al., 2013).

A decrease in stomatal conductance or an increase in carboxylation rates can result in increasing WUE, i.e. reduced leaf intercellular CO_2 concentration (C_i), and consequently carbon isotope discrimination ($\Delta^{13}C$) will decrease. Thus, there should be a negative correlation between WUE and $\Delta^{13}C$ because both parameters depend on stomatal aperture (Farquhar and Sharkey, 1982; Farquhar et al., 1989). In breeding programs, an integrated measurement of CO_2 assimilation to stomatal conductance or transpiration rate (i.e. losing water) could be reliably exploited by indirect selecting the superior genotypes that have high WUE through $\Delta^{13}C$ (Farquhar and Richards, 1984; Condon and Richards, 1992; Condon et al., 2002, 2004; Moghaddam et al., 2013). This discrimination would be least in those plants that exhibited the higher WUE (Dadkhah, 2013). In other words, genotypes with lower $\Delta^{13}C$ can be more efficient, in terms of water consumption if comparing with the high $\Delta^{13}C$ ones, under salinity conditions (Farquhar et al., 1989).

Iran is a part of the secondary center of origin of melons (*Cucumis melo* L.) in the world (Rubatzky and Yamaguchi, 1997), and various groups of them, generally, are cultivated in arid and semi-arid regions of the country with salinity problems. Iran ranks third in melon production after China and Turkey, with an annual production of 1.59 million tons from 78965 ha (FAO, 2017). Although melon is considered as particularly sensitive, or moderately sensitive, to the presence of NaCl in the root zone, limited evidence is available on the basic mechanisms of salinity responses associated with both stomatal and non-stomatal components of photosynthesis and especially carbon isotope discrimination. Therefore, this study was undertaken to test the hypothesis that $\Delta^{13}C$ is an important criterion of salinity stress in melon and the photosynthetic rate is directly related to $\Delta^{13}C$. The specific objectives of this study were:

(*i*) to assess the role of relative changes in stomatal and non-stomatal (e.g. Rubisco activity and, the integrity of the photochemical apparatus) limitations on photosynthetic capacity in the adaptation to adverse environmental conditions.
(*ii*) to further illuminate the possibilities of combining carbon stable isotope composition, as a breeding character, and conventional measurements of photosynthetic performance (gas exchange parameters) to select for higher WUE melon genotypes under saline conditions.
(*iii*) to evaluate the use of an optical sensor, DUALEX, of leaf chlorophyll and flavonol contents to assess crop nitrogen status of melons under salinity condition.
(*IV*) to test the theory that polyphenols, as secondary metabolites, are stimulated in plant tissues to counteract oxidative stress generated by salinity and contribute to stress tolerance.

Also, we considered the effects of salt concentrations on a variety of important physiological traits including nitrate reductase, ABA, osmotic potential etc.

By addressing these questions, new insight is expected from the study of carbon isotope discrimination, in order to enhance the efficiency of breeding efforts aiming to improve photosynthetic capacity of this crop. Furthermore, $\Delta^{13}C$ might be turned into an important proxy component for indirect selection of melons with tolerance to salinity.

2. Materials and methods

2.1. Plant material and growth conditions

This experiment was conducted during February and March 2016 in a greenhouse at the University of Paris-Sud (48°41′ N 2°11′E, Orsay, France) with average day temperatures between 25 and 33 °C and night temperatures between 15 and 20 °C with 55–65% relative humidity. Plants were grown under natural light conditions complemented with artificial light (high-pressure sodium bulb, Hortilux SchrEder B.V., Netherlands) during the day. The daily average of photosynthetic active radiation was 637 ± 45 μmol m^{-2} s^{-1} at the level of the plant canopy. Two commercial Iranian melon genotypes, "Suski-Sabz" and "Ghobadlu", from Eyvanki (35°20′N 52°04′E, Semnan Province) and Ajab Shir (37°28′N 45°53′E, East Azerbaijan Province), respectively, and "Galia" F1 (New American Seed Company, USA) were used for the experiments. Iranian melon genotypes are well known and popular Iranian local melon, which are under cultivation for hundreds of years and have genetic stability in most traits. Our previous study (Sarabi et al., 2017) clearly showed that these genotypes had a suitable performance in terms of higher tolerance to salt stress, in comparing with other genotypes studied, and therefore they have chosen for this experiment. "Galia" F1, as a salt-tolerant cultivar, was considered for comparison (Yasar et al., 2006).

Seeds were surface sterilized using 1% sodium hypochlorite with tween-20 as a surfactant for 10 min, then repeatedly washed with distilled water, and soaked for 12 h in distilled water. Then they were germinated on filter paper in Petri dishes moistened with sterile distilled water and transferred, after plant emergence, into plastic pots containing 4 L of substrate (perlite:vermiculite = 1:1 *v/v*) in the greenhouse. Plants were allowed to become established under non-saline conditions for the first 30 days of growth. The basic nutrient solution was a modified half-strength Hoagland solution. All chemicals used were of analytical grade with an electrical conductivity (EC) of 1.84 dS m^{-1}. On March 1, 2016, when plants had 4-5 expanded true leaves and produced male flowers, salinity treatments were conducted for the corresponding containers. The salt treatments were imposed in increments of 25 mM NaCl per day, to avoid osmotic shock, until the final concentrations of 50 and 100 mM were reached. The saline nutrient solutions had the same basic composition plus additional 50 and 100 mM of NaCl, giving EC values of 6.61 and 11.20 dS m^{-1}, respectively. The pH of the nutrient solutions for all treatments was adjusted to 6.0 ± 0.3 with H_2SO_4. All nutrient solutions were prepared using deionized water. All solutions were applied to plants by hand-watering on a daily basis. 15 and 30 days after treatments (DAT), the youngest fully expanded leaves were sampled, with three replicates per treatment and genotype, and then immediately frozen in liquid nitrogen and stored at −80 °C for biochemical analysis. Also, all plant tissues (including stem, leave, petiole and root) were harvested, and dried in a forced-air oven at 70 °C for 72 h for biomass determination.

2.2. Determination of leaf water relations

Leaf relative water content (RWC) was determined according to the method of Jones and Turner (1978), by using the following equation:

$$\mathrm{RWC}\ (\%) = \frac{FW - DW}{TW - DW} \times 100 \tag{2}$$

where, FW is the fresh weight at sampling time, DW the dry weight, and TW the turgid weight (saturated in water for one night). The dry weight of leaves was obtained after 24 h at 70 °C.

To calculate the osmotic potential, (Ψ_S), the most recent fully expanded leaves were put into plastic bags and rapidly frozen with liquid nitrogen. They were subsequently thawed and pressed to extract the cell sap. The sap was centrifuged at 15,000 g for 15 min at 4 °C, then the supernatant was utilized to determine osmolality using a cryo-osmometer (Camlab, Micro digital osmometer, Type 15, Cambridge, UK) calibrated using standard sugar solutions. The osmotic potential was determined according to the Van't Hoff equation (Nobel, 1991) as follows:

$$\Psi_S\ (\mathrm{MPa}) = -(C/1000)RT \tag{3}$$

Where, C is the osmomolarity (mM) in the leaf samples measured by cryo-osmometer, $R = 0.0083$ (MPa L mol^{-1} °K^{-1}) and T is the air temperature during the experiment (25 + 273 = 298 K).

2.3. Measurement of leaf gas exchange parameters, water use efficiency and chlorophyll fluorescence

After 15 and 30 days of salinity treatments, an open-flow gas-exchange system (LI-6400, LI-COR INC. Lincoln, Nebraska, USA) with the 6400-02B red and blue light source standard chamber was used for leaf gas exchange measurements on most recently fully expanded leaves between 10:00 and 16:00. Net photosynthesis (A_n), stomatal conductance to water vapour diffusion (g_s), transpiration (E) and intercellular to ambient CO_2 concentration ratio (C_i/C_a) were measured under ambient CO_2 concentration (400 μmol mol^{-1}) at a photosynthetic photon flux density (PPFD) of 1000 μmol photons m^{-2} s^{-1} and air temperature in the chamber of 25 °C. The air flow rate in the chamber was 400 μmol s^{-1} and the leaf-to-air vapour pressure deficit (VPD) was maintained at about 1 ± 0.1 kPa. Each leaf was allowed to reach a steady state of CO_2 uptake in the LI-6400 leaf chamber before measurements were taken. Intrinsic water use efficiency (WUE_i) was estimated as A_n/g_s, and instantaneous water use efficiency (WUE) was calculated as the ratio of net photosynthesis (A_n) and transpiration (E).

Measurements of chlorophyll fluorescence were conducted on the same leaf for gas exchange measurements using a portable chlorophyll fluorometer (PAM-2000, WALZ, Effeltrich, Germany). The leaves were dark-adapted for 30 min, so that all reaction centers of photosystem II (PSII) acquired an ‘open’ status, i.e. their electron acceptors QA were oxidized and heat loss was minimal (Strasser et al., 2000). Minimum fluorescence (F_0), maximum fluorescence (F_m), and maximum quantum efficiency of PSII photochemistry (Fv/Fm) were assessed. The variable fluorescence (F_v) was calculated from the difference between F_m and F_0.

2.4. *Optical sensor measurements*

Estimation of leaf chlorophyll and epidermal flavonol contents were made with hand-held leaf-clip devices, DUALEX 4 (Cerovic et al., 2012; Padilla et al., 2014). For individual measurements, the DUALEX measures a leaf surface area of 20 mm^2. Measurements with this device conducted on the same day for photosynthetic gas exchanges (15 and 30 DAT), using three plants in each replicate and value for each plant was the average of the six measurements. In each plant, assessment was done on the most recently fully expanded and well-lit leaf, on the distal part of the adaxial side of the leaf, midway between the margin and the mid-rib of the leaf. The nitrogen balance index (NBI) was also calculated as the ratio of the estimations of leaf chlorophyll to flavonoid contents (Padilla et al., 2014; Cerovic et al., 2015).

2.5. *Determination of rubisco activity (E.C. 4.1.1.39)*

Frozen leaf tissue (around 100 mg) were ground in a chilled mortar with quartz sand until obtaining a fine powder in the presence of liquid N_2, ice-cold 100 mM HEPES-KOH buffer, pH 7.5, containing 5 mM $MgCl_2$, 5 mM EGTA, 7 mM dithiothreitol (DTT), 6% PVP-25, 7% PEG 20000, 10% (*v/v*) glycerol and 1 μM leupeptine. After centrifugation at 18 000 *g* for 30 min at 4 °C, the supernatant was collected and used as enzymatic extract, after desalting extracts by centrifugal filtration on a Sephadex G25 gel (Amersham Biosciences). The assay buffer consisted of 100 mM Bicine pH 8.0, 25 mM $NaHCO_3$, 20 mM $MgCl_2$, 5 mM ceratine phosphate, 5 U creatine phosphokinase (CPK, Sigma), 5 U 3-phosphoglyceric phosphokinase (3PGK, Sigma), 5 U glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Sigma), 0.25 mM nicotinamide adenine dinucleotide (NADH, Sigma) and 3.5 mM adenosine triphosphate (ATP, Sigma). For the initial activity, 80 μl of extract was added in 720 μl of assay buffer and the reaction was initiated by adding 40 μl of 10 mM ribulose-1,5-bisphosphate (RuBP, Sigma). For the total activity, the extract was incubated at 25 °C in the assay buffer for 10 min and served to activate any unactivated Rubisco in the assay solution (Dann and Pell, 1989). The Rubisco activity was determined by monitoring the oxidation rate of NADH, by assuming two molecules of NADH oxidized per molecule of CO_2 fixed (Zahoor et al., 2017; Sehar et al., 2019). The change in absorbance at 340 nm was monitored for 4 min. Both the initial and total activities were expressed as μmol CO_2 min^{-1} mg^{-1} protein. Rubisco activation state or percent activation was calculated by the ratio of initial to total Rubisco activities (Crafts-Brandner and Salvucci, 2000). For each treatment, extracts from three distinct leaves were evaluated, each in duplicate.

2.6. *Nitrate reductase (E.C. 1.6.6.1) assay*

Leaf samples (around 100 mg) were ground in liquid nitrogen and the resulting powder dissolved in 1 ml of 50 mM HEPES-NaOH buffer, pH 7.6, containing either 0.1% Triton X-100, 5 mM $MgCl_2$, 10 μM leupeptin, 0.5 mM AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride], 3 mM DTT, 1% polyvinylpolypyrrolidone, 3% polyethylene glycol 4000 and 19.2 μM flavine adenine dinucleotide (FAD). This was followed by centrifugation for 10 min at 13 000 *g* (Fresneau et al., 2007). Measurements of total (in the presence of EDTA (ethylenediaminetetraacetic acid), NR-EDTA) and active (actual) (in the presence of Mg^{2+}, NR-Mg) NR activities were carried out on the supernatant following the method described by Foyer et al. (1998). Briefly, the reaction mixture contained 250 μl of 50 M HEPES-NaOH buffer, pH 7.6, 50 μl of 100 mM KNO_3, and 50 μl of 2 mM nicotinamide adenine dinucletide (NADH). The reaction was initiated by adding 50 μl of extract and incubated at 30 °C for 30 min. The reaction was stopped by adding 50 μl of 1 M $ZnSO_4$ 7 H_2O. The nitrite ions produced we assayed after diazotization with 500 μl of 1.5% (*w/v*) sulfanilamide and 500 μl of 0.02% (*w/v*) N-(1-Naphthyl) ethyl-Enediamine (NEDD). Then, the solution was again centrifuged at 8000 *g* for 10 min and the absorbance of the supernatant was measured at 540 nm. The catalytic activity of both NR-EDTA and NR-Mg were estimated as μmol NO_2^- formation per mg protein and per hour. The activation percent of NR was determined by the ratio between the NR-Mg to NR-EDTA.

2.7. *Protein content determination*

The protein amount in all enzymatic extracts was determined by Bradford's method (1976). This allowed all enzymatic activities to be expressed relative to the soluble protein concentration.

2.8. *ABA extraction and quantification*

Leaf ABA was extracted and purified according to the method described by Dobrev and Kaminek (2002), and its content was determined by high-performance liquid chromatography (HPLC) as described by Albacete et al. (2008).

2.9. *Carbon isotope discrimination analyses*

After freeze-drying of leaf samples (three replicates per treatment and genotype), they were finely ground with a ball mill (Retsch MM200, Bioblock Scientific, Illkirch, France) to obtain a fine powder. Then, an aliquot of 500–600 μg per sample powder were packed in tin capsules (Courtage Analyse Service, Mont Saint-Aignan, France) and used for ^{13}C analysis with an elemental analyzer (Flash A 1112, ThermoFicher, Germany) coupled to an isotope ratio mass spectrometer (Deltaplus^{XP} IRMS Thermo-Finnigan, Germany) via a ConFlo III interface (Finnigan MAT, Germany), as described by Werner et al. (1999), at the Grassland Science Laboratory of ETH-Zurich (Switzerland). Carbon isotope composition ($\delta^{13}C$) was expressed as deviation of the carbon isotope ratio ($^{13}C/^{12}C$, called R) from the international standard (Vienna Pee Dee Belemnite limestone, V-PDB):

$$\delta^{13}C\,(0) = [(\frac{R_{sample} - R_{V-PDB}}{R_{V-PDB}})] \times 1000 \tag{4}$$

Two international isotope secondary standards of known $^{13}C/^{12}C$ ratios (IAEA l-glutamic acid and IAEA sucrose), and one laboratory standard (glutamic acid) were measured in order to check for the possible drift of the IRMS (one capsule of laboratory glutamic acid every six samples, and two capsules of each IAEA standard every 24 samples). The precision of the IRMS measurements was 0.1‰. Carbon isotope discrimination ($\Delta^{13}C$) was calculated according to Farquhar et al. (1989):

$$\Delta^{13}C\,(0) = [\frac{(\delta^{13}C_a - \delta^{13}C_p)}{(\delta^{13}C_p + 1)}] \tag{5}$$

where, $\delta^{13}C_p$ is the carbon isotope composition of the plant organic matter, as photosynthetic product, and $\delta^{13}C_a$ that of the atmospheric CO_2, as carbon source, (which was −9.5‰ in the greenhouse).

2.10. *Data analysis and statistics*

The treatments and genotypes were arranged in a factorial experiment as a completely randomized design with three replicates per treatment and four plants were used in each replication. Genotypes and salinity treatments were used as independent factors and subjected to Analysis of Variance (ANOVA). Duncan's Multiple Range Test at a confidence level of 5% ($P < 0.05$) was carried out as the post-hoc test to find significant differences across salinity treatments within each genotype. Trends were considered significant when the mean values of the compared sets differed at $P < 0.05$. All statistical tests were performed using the computer program PASW statistics 18.0 (SPSS Inc., Chicago, IL, USA). A bivariate correlation procedure was used to calculate the Pearson's correlation coefficients between the measured attributes across all treatment levels (0, 50 and 100 mM NaCl) and genotypes. Moreover, multiple linear regression analysis (stepwise) was performed on the data from two sampling times (15 and 30 DAT), using photosynthesis (A_n) as a dependent variable, and other traits studied as independent variables.

3. Results

3.1. RWC and Ψ_S

Increasing concentrations of NaCl from 0 to 100 mM significantly decreased the leaf RWC of melon plants at both 15 and 30 DAT ($P < 0.001$, Table 1) with no significant difference among genotypes. When averaged all genotypes, the RWC was significantly lower by 13% and 9% at 100 mM NaCl compared to control at 15 and 30 DAT, respectively (Table 1).

Statistical analysis of leaf Ψ_S showed a significant difference among salt treatments and among the genotypes at two sampling times with no significant difference on the salinity × genotype interaction at second date (Table 1). The average of the three genotypes revealed a decrease in Ψ_S by 27% and 33% at 15 DAT and 28% and 33% at 30 DAT under 50 and 100 mM treatments, respectively, when compared with their controls. However, the rate of this reduction differed among genotypes and such decreases were greatest in “Ghobadlu” (39%) and “Galia” F1 (35%) at 15 and 30 DAT, respectively (Table 1). Also the maximum Ψ_S value (less negative) was detected in “Suski-e-Sabz” at 15 and 30 DAT.

3.2. Leaf area, specific leaf area and biomass

In sampling date 1, the leaf area (LA) was significantly affected by salinity treatment, genotype, and their interaction, whereas there were no significant genotype difference and salinity × genotype interaction at sampling date 2 (Table 1). Compared with control plants (0 mM NaCl), a significant decrease of 18% and 30% in LA was measured in melon plants in response to 100 mM NaCl treatment at 15 and 30 DAT, respectively ($P < 0.001$). The change rates were different among genotypes and the lowest rates were found in “Galia” F1 at both sampling times (Table 1). At 15 DAT, the maximum value of LA was recorded in “Suski-e-Sabz”, followed by “Ghobadlu”, and the lowest value was observed in “Galia” F1 ($P < 0.05$, Table 1), while no considerable difference observed among genotypes at the second sampling time.

Salinity treatments inhibited the specific leaf area (SLA) of all melon genotypes, leading to a significant reduction in SLA (-of 27%) at 100 mM NaCl, as compared to the control treatments at 15 and 30 DAT, however, no significant differences between 50 and 100 mM were observed (Table 1). When saline treatments are averaged, “Galia” F1 significantly exhibited the lowest SLA at both sampling times (Table 1).

As the concentrations of NaCl in the nutrient solution increased, the biomass significantly decreased in three genotypes ($P < 0.01$ and $P < 0.001$ at 15 and 30 DAT; respectively, Table 1). Compared with control treatment, the 50 and 100 mM NaCl resulted in a significant decrease in plant biomass, respectively by 26% and 34% at 15 DAT and by 21% and 39% for 30 DAT (Table 1). The values of the biomass were higher in “Galia” F1 than those in the two other genotypes, when averaged for all salt treatments at two sampling times.

Table 1
Effects of NaCl treatments on relative water content (RWC), osmotic potential (Ψ_S), specific leaf area (SLA), leaf area (LA), and biomass across melons studied at 15 and 30 days after treatments (15 and 30 DAT).

	RWC (%)		Ψ_S (MPa)		SLA ($cm^2\ g^{-1}$)		LA (m^2 per plant)		Biomass (g)	
Treatments	15 DAT	30 DAT	15 DAT	30 DAT	15 DAT	30 DAT	15 DAT	30 DAT	15 DAT	30 DAT
Galia F1										
Control	88.39 ± 2.42 ab	89.63 ± 1.93 a	−0.79 ± 0.035 ab	−0.75 ± 0.031 a	463.5 ± 29.78 b-d	444.7 ± 21.98 b-d	3.32 ± 0.10 bc	5.20 ± 0.09 a	15.55 ± 0.97 a	26.77 ± 1.26 a
50 mM NaCl	82.48 ± 1.27 cd	80.4 ± 1.81 b	−1.03 ± 0.003 cd	−1.01 ± 0.058 c-e	336.8 ± 19.39 d	284.3 ± 10.24 d	3.34 ± 0.05 bc	4.24 ± 0.08 bc	11.15 ± 0.34 b-d	20.46 ± 1.03 b
100 mM NaCl	78 ± 1.81 de	82.34 ± 1.19 b	−1.21 ± 0.058 f	−1.16 ± 0.018 e	333.1 ± 9.20 d	308.4 ± 6.06 cd	2.79 ± 0.06 d	3.69 ± 0.13 d	9.693 ± 0.65 cd	17.06 ± 0.37 c
Ghobadlu										
Control	92.46 ± 1.37 a	89.22 ± 0.84 a	−0.70 ± 0.047 a	−0.78 ± 0.032 ab	659.1 ± 6.51 a	696.5 ± 66.90 a	3.53 ± 0.06 ab	5.32 ± 0.11 a	14.32 ± 0.18 ab	23.69 ± 1.43 ab
50 mM NaCl	82.44 ± 2.57 cd	82.97 ± 1.06 b	−1.19 ± 0.029 ef	−1.11 ± 0.076 de	429.8 ± 60.22 cd	513.5 ± 72.85 b	3.17 ± 0.06 c	4.41 ± 0.17 b	11.36 ± 0.77 a-d	22.22 ± 0.98 b
100 mM NaCl	77.22 ± 1.06 de	80.91 ± 0.79 b	−1.14 ± 0.020 d-f	−1.13 ± 0.046 e	501.7 ± 72.12 bc	463.8 ± 25.25 bc	2.86 ± 0.03 d	3.69 ± 0.18 d	10.04 ± 0.60 cd	15.96 ± 0.57 c
Suski-e-Sabz										
Control	86.6 ± 2.30 bc	88.62 ± 0.53 a	−0.79 ± 0.063 ab	−0.66 ± 0.036 a	585 ± 59.45 ab	524.8 ± 62.96 b	3.69 ± 0.11 a	5.60 ± 0.20 a	13.27 ± 3.39 a-c	21.22 ± 1.73 b
50 mM NaCl	78.67 ± 1.59 de	80.19 ± 1.35 b	−0.92 ± 0.080 bc	−0.92 ± 0.070 bc	549.5 ± 40.91 a-c	363.6 ± 54.05 b-d	3.28 ± 0.05 c	4.14 ± 0.06 bc	9.21 ± 0.72 cd	13.76 ± 1.03 cd
100 mM NaCl	75.74 ± 1.28 e	79.71 ± 1.21 b	−1.04 ± 0.043 c-e	−0.97 ± 0.010 cd	406.4 ± 53.74 cd	447.4 ± 69.69 b-d	2.95 ± 0.04 d	3.90 ± 0.05 cd	8.753 ± 0.92 d	11 ± 0.43 d
Salinity treatments (S)	***	***	***	***	**	**	***	***	**	***
Genotypes (G)	NS	NS	*	**	**	***	*	NS	NS	***
G × S	NS	NS	*	NS	NS	NS	*	NS	NS	NS

Values are means ± standard error of three replicates. Means followed by different letters in the same column are significantly different ($P < 0.05$, Duncan's multiple range tests).
Significance levels are expressed as: NS when $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 2

Effects of NaCl treatments on leaf chlorophyll content, flavonols content, nitrogen balance index (NBI), activation percent of NR (%), and abscisic acid (ABA) concentration across melons studied at 15 and 30 days after treatments (15 and 30 DAT).

Treatments	Chlorophyll (DUALEX units)		Flavonols (DUALEX units)		NBI (Chlorophyll DUALEX/Flavonols DUALEX)		Activation percent of NR (%)		ABA (ng/g DW)	
	15 DAT	30 DAT	15 DAT	30 DAT	15 DAT	30 DAT	15 DAT	30 DAT	15 DAT	30 DAT
Galia F1										
Control	33.9 ± 0.61 a	34.67 ± 0.48 a	0.900 ± 0.082 ab	0.820 ± 0.072 ab	38.15 ± 2.65 bc	42.83 ± 3.12 b	72.72 ± 0.691 b	89.77 ± 1.09 a	139 ± 20.63 ab	129.6 ± 18.66 c
50 mM NaCl	29.7 ± 0.68 b	33.37 ± 0.94 ab	1.003 ± 0.043 ab	0.977 ± 0.091 a	29.69 ± 1.20 de	34.65 ± 2.68 b-d	66.57 ± 1.051 c	58.75 ± 1.82 c	281.1 ± 59.29 ab	342.1 ± 43.41 bc
100 mM NaCl	29.3 ± 0.90 bc	25.03 ± 1.08 d	1.020 ± 0.040 a	0.990 ± 0.027 a	28.78 ± 1.03 de	25.28 ± 0.74 de	56.24 ± 1.389 de	53.61 ± 1.74 de	835 ± 65.68 a	453.2 ± 36.12 bc
Ghobadlu										
Control	26.13 ± 0.77 de	31.77 ± 1.41 ab	0.557 ± 0.029 d	0.620 ± 0.057 b	47.09 ± 1.60 a	52.52 ± 7.27 a	86.47 ± 1.099 a	88.13 ± 2.32 a	532 ± 62.70 a	331.3 ± 39.03 bc
50 mM NaCl	24.63 ± 0.74 de	27.23 ± 1.05 cd	0.597 ± 0.043 d	0.690 ± 0.035 b	41.53 ± 1.80 ab	39.63 ± 2.23 bc	73.58 ± 1.885 b	65.8 ± 2.03 b	1067.9 ± 554.24 a	592.7 ± 27.11 ab
100 mM NaCl	23.57 ± 1.01 e	26.6 ± 1.10 d	0.677 ± 0.043 cd	0.743 ± 0.088 b	34.95 ± 1.40 cd	36.42 ± 2.69 bc	59.13 ± 1.112 d	57.72 ± 1.25 cd	568 ± 21.44 a	702.6 ± 31.28 ab
Suski-e-Sabz										
Control	26.7 ± 0.95 cd	30.13 ± 1.60 bc	0.800 ± 0.103 bc	0.963 ± 0.048 a	34.31 ± 3.62 cd	31.37 ± 1.65 cd	56.35 ± 1.496 de	60.76 ± 1.41 c	882.4 ± 648.33 a	133.7 ± 22.80 c
50 mM NaCl	26.93 ± 1.08 cd	26.13 ± 0.88 d	0.913 ± 0.069 ab	0.997 ± 0.044 a	21.04 ± 2.34 f	26.25 ± 0.27 de	53.4 ± 1.046 e	49.45 ± 0.64 e	515.6 ± 30.22 a	945.5 ± 44.86 a
100 mM NaCl	18.9 ± 0.84 f	19.07 ± 0.78 e	1.000 ± 0.076 ab	1.000 ± 0.070 a	27.09 ± 1.11 e	19.15 ± 0.62 e	42.74 ± 1.470 f	40.01 ± 1.6 f	623.5 ± 46.49 a	1032 ± 58.21 a
Significance										
Salinity treatments (S)	***	***	*	NS	***	***	***	***	NS	***
Genotypes (G)	***	***	***	***	***	***	***	***	NS	**
G × S	**	*	NS	NS	NS	NS	**	***	NS	NS

Values are means ± standard error of three replicates. Means followed by different letters in the same column are significantly different ($P < 0.05$, Duncan's multiple range tests).
Significance levels are expressed as: NS when $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 3

Effects of NaCl treatments on photosynthetic rate (A_n), stomatal conductance to water vapour diffusion (g_s), transpiration rate (E), internal to ambient CO_2 concentration ratio (C_i/C_a) and chlorophyll fluorescence (Fv/Fm) across melon studied at 15 and 30 days after treatments (15 and 30 DAT).

Treatments	A_n (μmol CO_2 m^{-2} s^{-1})		g_s (mol H_2O m^{-2} s^{-1})		E (mmol H_2O m^{-2} s^{-1})		C_i/C_a		Fv/Fm	
	15 DAT	30 DAT	15 DAT	30 DAT	15 DAT	30 DAT	15 DAT	30 DAT	15 DAT	30 DAT
Galia F1										
Control	13.5 ± 0.12 a	12.60 ± 0.38 a	0.183 ± 0.015 a	0.190 ± 0.005 a	2.72 ± 0.154 a	2.127 ± 0.046 b	0.625 ± 0.048 a	0.640 ± 0.033 ab	0.814 ± 0.008 a	0.815 ± 0.003 a
50 mM NaCl	10.22 ± 0.44 b	10.86 ± 0.70 b	0.093 ± 0.004 d	0.084 ± 0.007 b	1.72 ± 0.059 cd	1.117 ± 0.068 e	0.534 ± 0.039 bc	0.530 ± 0.029 b-d	0.811 ± 0.008 ab	0.802 ± 0.003 d
100 mM NaCl	8.853 ± 0.23 b	8.84 ± 0.30 c	0.067 ± 0.007 f	0.049 ± 0.002 b	1.29 ± 0.083 f	0.852 ± 0.090 e	0.397 ± 0.036 e	0.406 ± 0.023 e	0.799 ± 0.013 de	0.797 ± 0.005 e
Ghobadlu										
Control	12.57 ± 0.55 a	12.47 ± 0.61 ab	0.165 ± 0.011 b	0.203 ± 0.044 a	2.24 ± 0.058 b	1.803 ± 0.144 c	0.526 ± 0.014 b-d	0.674 ± 0.017 a	0.806 ± 0.002 bc	0.810 ± 0.005 b
50 mM NaCl	10.04 ± 0.39 b	10.77 ± 1.06 b	0.084 ± 0.004 e	0.148 ± 0.014 a	1.59 ± 0.072 d-f	1.423 ± 0.085 d	0.480 ± 0.012 cd	0.506 ± 0.037 c-e	0.802 ± 0.002 cd	0.806 ± 0.006 c
100 mM NaCl	9.81 ± 0.35 b	8.91 ± 0.07 c	0.086 ± 0.007 e	0.077 ± 0.004 b	1.59 ± 0.054 d-f	1.143 ± 0.029 e	0.448 ± 0.013 de	0.440 ± 0.041 de	0.802 ± 0.007 cd	0.794 ± 0.011 f
Suski-e-Sabz										
Control	10.08 ± 0.51 b	12.77 ± 0.59 a	0.143 ± 0.014 c	0.187 ± 0.025 a	1.91 ± 0.116 c	2.460 ± 0.124 a	0.576 ± 0.012 ab	0.660 ± 0.060 a	0.805 ± 0.004 c	0.808 ± 0.004 c
50 mM NaCl	9.553 ± 0.54 b	9.05 ± 0.23 c	0.093 ± 0.013 d	0.173 ± 0.016 a	1.63 ± 0.162 c-e	1.710 ± 0.111 c	0.495 ± 0.021 b-d	0.609 ± 0.025 a-c	0.795 ± 0.010 ef	0.790 ± 0.004 g
100 mM NaCl	9.703 ± 0.42 b	6.27 ± 0.39 d	0.071 ± 0.005 f	0.081 ± 0.008 b	1.37 ± 0.075 ef	1.092 ± 0.087 e	0.389 ± 0.014 e	0.511 ± 0.046 c-e	0.791 ± 0.002 f	0.782 ± 0.005 h
Salinity treatments (S)	***	***	***	***	***	***	***	***	NS	**
Genotypes (G)	**	**	NS	*	*	***	NS	NS	NS	*
G × S	**	NS	NS	NS	**	**	NS	NS	NS	NS

Values are means ± standard error of three replicates. Means followed by different letters in the same column are significantly different ($P < 0.05$, Duncan's multiple range tests). Significance levels are expressed as: NS when $P > 0.05$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.3. *Responses of optical sensor measurements to salinity treatments*

The effects of salt stress on chlorophyll and flavonol contents and NBI are shown in Table 2. In both sampling dates, the chlorophyll content was significantly affected by salinity, genotypes and their interaction. When three genotypes were averaged, the chlorophyll content significantly decreased by 17% and 27% under 100 mM NaCl at 15 and 30 DAT, respectively, as compared with their controls ($P < 0.001$, Table 2). However, the rate of this decrease differed between genotypes and the highest decrease was detected in “Suski-e-Sabz” (29% and 37% at 15 and 30 DAT, respectively). Across all saline treatments considered, the highest chlorophyll content was recorded in “Galia” F1 for both sampling dates ($P < 0.001$, Table 2).

Differences in leaf flavonol content between salt treatments was apparent at 15 DAT ($P < 0.05$, Table 2), averaged three melon genotypes. However, at 30 DAT there was no significant difference in flavonol content between treatments. For all melon genotypes studied, there was a consistent general pattern in the increase in flavonol content at 15 and 30 DAT, across all NaCl treatments. Such that flavonol content was elevated to 10% and 16% at 15 DAT and to 10% and 12% at 30 DAT under 50 and 100 mM NaCl, respectively, as compared to their controls (Table 2). At two sampling dates, the lowest flavonol content was detected in “Galia” F1 ($P < 0.001$, Table 2).

On average of the three genotypes, NBI values were sensitive to NaCl concentrations and significantly declined by 23%, 24% at 15 DAT and by 21%, 36% at 30 DAT under 50 and 100 mM NaCl, compared with their controls, respectively ($P < 0.001$, Table). However the rate of this decrease differed according to the genotypes and it can be noted that the greatest rate of reduction of NBI was observed in “Galia” F1 at 30 DAT (41%) under 100 mM NaCl compared with control condition (Table 2). NBI average significantly varied among melons ($P < 0.001$, Table 2) and “Ghobadlu” had the highest NBI whereas “Suski-e-Sabz” had the lowest value, across all saline treatments at two sampling date.

3.4. *NR*

At both sampling times, the activation percent of NR was significantly affected by salinity treatment, genotype, and their interaction (Table 2). This parameter exhibited notable decline in response to increasing concentrations of NaCl at 15 and 30 DAT ($P < 0.001$). However, the maximum decline was observed at the concentration of 100 mM NaCl in “Galia” F1 at 30 DAT and was reduced by 40%, if compared to the control. NR enzyme activity was different according to genotypes ($P < 0.001$), such that the highest and lowest NR activities were detected in “Ghobadlu” and “Suski-e-Sabz”, respectively, at both sampling dates.

3.5. *ABA*

Increasing salt concentrations in the nutrient solution, increased ABA content by 17%, 23% at 15 DAT ($P > 0.05$, Table 2) and by 3.2, 3.7 fold at 30 DAT ($P < 0.001$, Table 2) under 50 and 100 mM NaCl, respectively, compared to their control conditions over all melon genotypes. Generally, the increase rates were different among genotypes and the greatest rates at 15 and 30 DAT were observed in “Galia” F1 (83%) and “Suski-e-Sabz” (87%), respectively, under 100 mM NaCl as compared with non-saline treatments (Table 2). Two weeks after salinization, there was no significant difference among genotypes, however at 30 DAT, ABA content significantly varied across the studied melons ($P < 0.01$, Table 2), ranged from 308.3 (ng/g DW) in “Galia” F1 to 703.7 (ng/g DW) in “Suski-e-Sabz”.

3.6. *Gas exchange parameters*

Net photosynthesis (A_n) of melons leaves in both sampling times was highly influenced by salinity and genotype, whereas no significant effect was observed on the salinity × genotype interaction at the second date (Table 3). Across all plants, the A_n decreased by 22% at 15 DAT and 37% at 30 DAT under 100 mM NaCl as compared with control plants. Net photosynthetic rate was significantly lower in "Suski-e-Sabz" with respect to other genotypes, averaged all treatments ($P < 0.01$). In addition, the greatest reduction rate (51%) was observed in "Suski-e-Sabz" at second sampling date under 100 mM treatment, compared with non-saline condition.

When averaged across genotypes, a significant decrease in g_s by 45% and 54% at 15 DAT and by 30% and 64% at 30 DAT was observed under 50 and 100 mM NaCl, respectively, in comparison to the control treatment (Table 3). At 15 and 30 DAT, "Galia" F1 had the greatest decrease in g_s among the genotypes (63% and 74%, respectively) under 100 mM NaCl treatment, with reference to control treatment. g_s did not show significant difference between genotypes at 15 DAT ($P > 0.05$, Table 3), while the lowest g_s value was observed in "Galia" F1 at 30 DAT ($P < 0.05$, Table 3).

Leaf transpiration (E) was significantly affected by salinity level, genotype, and their interaction at two sampling date (Table 3). As it shown in Table 3, E decreased by 28% and 38% at 15 DAT and by 33% and 52% at 30 DAT under 50 and 100 mM NaCl, respectively, as compared to control treatment ($P < 0.001$, Table 3). However, the rate of the decrease differed among genotypes and it can be noted that "Galia" F1 showed the highest change rates at both sampling dates (53% and 60% at 15 and 30 DAT, respectively, Table 3) under 100 mM NaCl in comparison with control plants. Of the three genotypes, the maximum transpiration was recorded in "Galia" F1 and "Suski-e-Sabz" at 15 and 30 DAT, respectively, averaging all salt treatments ($P < 0.05$ and $P < 0.001$, Table 3, respectively).

The significant reduction of C_i/C_a value in the 100 mM NaCl treatment, with reference to controls, was 29% at 15 DAT and 31% at 30 DAT, respectively ($P < 0.001$, Table 3). Higher C_i/C_a values were detected in "Galia" F1 and "Suski-e-Sabz" at 15 and 30 DAT, respectively, but differences were not significant ($P > 0.05$, Table 3). Also the greatest decrease rates in C_i/C_a values were found in "Galia" F1 at first and second sampling times (36% and 37% decrease, respectively, with respect to controls) under 100 mM NaCl concentration.

3.7. Photochemical efficiency of PSII

Salinity treatments had no major impact on the maximum photochemical efficiency of PSII at 15 DAT, with Fv/Fm values of all melons studied being within 0.797–0.808 range and not significantly different from each other ($P < 0.05$). While, increasing NaCl concentrations from 50 to 100 mM, significantly decreased the Fv/Fm values at 30 DAT, when averaged over genotypes ($P < 0.01$, Table 3), with the lowest Fv/Fm ratio recorded in "Suski-e-Sabz" across salt treatments ($P < 0.05$, Table 3). Moreover, the highest Fv/Fm values were recorded in "Galia" F1 compared to other melon genotypes studied at 15 and 30 DAT when averaged over salinity treatments.

3.8. Water use efficiency

There was no significant difference in WUE and WUE_i among genotypes at 15 DAT, while at 30 DAT, WUE and WUE_i were significantly different among melons and "Galia" F1 demonstrated the highest values across all NaCl concentrations (8.8 μmol CO_2 $mmol^{-1}$ H_2O and 125.3 μmol CO_2 mol^{-1} H_2O, respectively, $P < 0.001$, Table 4). Increased salinity levels resulted in improved photosynthetic water use efficiency in melon plants grown under 50 and 100 mM of NaCl (Table 4). Such that WUE and WUE_i were significantly increased by 21% and 42% at 15 DAT and by 25% and 44% at 30 DAT, under 100 mM NaCl, respectively, as compared to their controls (Table 4). However, the rate of the increase differed among genotypes. Such that, "Suski-e-Sabz" exhibited the greatest increment rates for both WUE and WUE_i at 15 DAT, while "Galia" F1 had the highest change rates at second sampling date for both parameters.

3.9. Initial and total rubisco activities

Initial and total Rubisco activities in fully expanded melon leaves at 15 and 30 DAT was highly affected by salinity, genotype, and their interaction (Table 4). Both the initial and total activities of this enzyme significantly decreased as a result of treatment with 50 or 100 mM NaCl after 15 and 30 days of commencing salt treatments ($P < 0.001$, Table 4). That is, the initial Rubisco activities of the three genotypes markedly decreased by 26% and 63% at 15 DAT, and by 39% and 62% at 30 DAT, under 50 and 100 mM NaCl, respectively, when compared with the control. In addition, total Rubisco activity declined by 23% and 58% at 15 DAT, and by 39% and 57% at 30 DAT for all melon studied under 50 and 100 mM salt solutions, respectively, compared with non-saline treatment. Significant genotype difference in the initial and total Rubisco activity responses to salinity stress was also observed after 15 and 30 days of salt treatments ($P < 0.001$, Table 4). "Galia" F1 had the highest initial Rubisco activity at both sampling dates (3.46 and 6.70 μmol CO_2 min^{-1} mg^{-1} protein, at 15 and 30 DAT, respectively). Moreover, "Ghobadlu" (4.48 μmol CO_2 min^{-1} mg^{-1} protein) and "Galia" F1 (8.05 μmol CO_2 min^{-1} mg^{-1} protein) exhibited the maximum total Rubisco activity at 15 and 30 DAT, respectively, over all salt treatments. On average of two sampling time, the initial Rubisco activity increased from 2.82 to 4.78 μmol CO_2 min^{-1} mg^{-1} protein, while total Rubisco activity increased from 3.79 to 5.98 μmol CO_2 min^{-1} mg^{-1} protein across all genotypes and also salt treatments.

In addition, as shown in Fig. 1, the general trend observed in the genotypes, with the exception of the "Ghobadlu" at second sampling time, was a decrease in activation state of Rubisco with increasing stress levels at 15 and 30 DAT ($P > 0.05$ and $P < 0.01$, respectively). Of the three genotypes, the highest and lowest activation rates were recorded in "Galia" F1 and "Suski-e-Sabz", respectively, when averaged over all NaCl concentrations for both sampling dates. Moreover, the activation state increased from 72.4% at 15 DAT to 77.9% at 30 DAT over all genotypes and all salinity treatments.

3.10. Carbon isotope discrimination

The data obtained from both sampling dates indicated that the $\Delta^{13}C$ of all melon genotypes significantly decreased by salt stress ($P < 0.001$, Table 4), changing from 20.7‰ to 17.1‰ at 15 DAT and from 18.9‰ to 15.4‰ at 30 DAT, for the least and the most saline treatments, averaged all genotypes. The greatest reduction rate of $\Delta^{13}C$ was observed in "Galia" F1 (5.27‰) and Suski-e-Sabz (4.69‰) at 15 and 30 DAT, respectively, under 100 mM NaCl as compared with control treatment. "Ghobadlu" and "Galia" F1 showed lower but not significant $\Delta^{13}C$ values at 15 and 30 DAT, respectively, across all saline treatments. Averaged all salt concentrations and all melon genotypes, $\Delta^{13}C$ decreased from 18.94‰ to 16.90‰ at first and second sampling date, respectively.

3.11. Multiple linear regression

Stepwise multiple linear regression (MLR) was performed to determine the most significant physiological traits contributing to photosynthesis performance (A_n) of melon genotypes studied under salinity stress at two sampling dates (Table 5). The results indicated that E, WUE, C_i, g_s, and Rubisco total activity mainly explain the variability in photosynthesis at 15 DAT. Based on this analysis, A_n was positively and significantly correlated with E ($P < 0.001$), WUE ($P < 0.001$), C_i ($P < 0.01$), Rubisco total activity ($P < 0.05$) and negatively with g_s ($P < 0.05$). The coefficient of determination of the MLR at first sampling date was 99% (significant at $P < 0.001$). Also, variations in A_n at 30 DAT are mostly accounted by positive Fv/Fm ($P < 0.01$) and C_i ($P < 0.01$), with $R^2 = 0.98$ ($P < 0.001$). The equations associated

Table 4

Effects of NaCl treatments on instantaneous water use efficiency (WUE), intrinsic water use efficiency (WUE_i), initial and total activities of Rubisco as well as carbon isotope discrimination ($\Delta^{13}C$) across melons studied at 15 and 30 days after treatments (15 and 30 DAT).

	WUE (μmol CO_2 $mmol^{-1}$ H_2O)		WUEi (μmol CO_2 mol^{-1} H_2O)		Rubisco initial activity (μmol CO_2 min^{-1} mg^{-1} protein)		Rubisco total activity (μmol CO_2 min^{-1} mg^{-1} protein)		$\Delta^{13}C$ (‰)	
Treatments	15 DAT	30 DAT	15 DAT	30 DAT	15 DAT	30 DAT	15 DAT	30 DAT	15 DAT	30 DAT
Galia F1										
Control	5.00 ± 0.303 e	5.93 ± 0.15 cd	74.75 ± 6.14 c	66.33 ± 1.46 c	5.05 ± 0.10 a	9.70 ± 0.38 a	6.12 ± 0.07 a	11.31 ± 0.55 a	22 ± 0.85 a	17.79 ± 1.11 bc
50 mM NaCl	5.93 ± 0.056 cd	9.74 ± 0.46 a	109.8 ± 3.98 b	130 ± 2.50 b	3.79 ± 0.29 b	5.65 ± 0.32 c	4.61 ± 0.18 b	7.05 ± 0.28 c	19 ± 1.66 bc	15.96 ± 1.04 cd
100 mM NaCl	6.91 ± 0.254 ab	10.61 ± 1.23 a	134 ± 9.18 a	179.6 ± 7.83 a	1.55 ± 0.20 d	4.75 ± 0.30 d	2.25 ± 0.37 de	5.79 ± 0.34 de	16.73 ± 0.01 c	15.02 ± 0.21 d
Ghobadlu										
Control	5.62 ± 0.328 c-e	6.95 ± 0.29 b-d	76.57 ± 2.44 c	70.1 ± 20.74 c	5.06 ± 0.06 a	7.18 ± 0.18 b	6.49 ± 0.24 a	9.29 ± 0.32 b	19.67 ± 1.33 a-c	18.46 ± 0.85 ab
50 mM NaCl	6.33 ± 0.113 bc	7.56 ± 0.48 bc	119 ± 1.05 ab	74.28 ± 10.42 c	3.17 ± 0.25 c	4.08 ± 0.17 de	4.69 ± 0.07 b	4.90 ± 0.26 ef	19.55 ± 0.59 a-c	17.28 ± 1.13 b-d
100 mM NaCl	6.19 ± 0.201 c	7.80 ± 0.14 b	115.6 ± 9.40 ab	116.6 ± 6.17 b	1.54 ± 0.18 d	2.41 ± 0.12 f	2.26 ± 0.20 de	2.89 ± 0.26 g	17.13 ± 0.13 c	15.45 ± 0.30 cd
Suski-e-Sabz										
Control	5.29 ± 0.146 de	5.23 ± 0.46 d	72.68 ± 10.28 c	72.05 ± 13.71 c	1.94 ± 0.05 d	4.72 ± 0.20 d	2.90 ± 0.08 c	5.93 ± 0.19 d	20.56 ± 0.63 ab	20.43 ± 0.54 a
50 mM NaCl	5.90 ± 0.232 cd	5.32 ± 0.23 d	105.6 ± 8.64 b	53.09 ± 4.46 c	1.95 ± 0.15 d	3.41 ± 0.19 e	2.72 ± 0.19 cd	4.31 ± 0.19 f	18.42 ± 0.91 bc	15.83 ± 0.50 cd
100 mM NaCl	7.11 ± 0.182 a	5.83 ± 0.63 cd	136.4 ± 4.12 a	78.43 ± 6.05 c	1.35 ± 0.26 d	1.14 ± 0.03 g	2.05 ± 0.14 e	2.35 ± 0.15 g	17.4 ± 0.34 c	15.74 ± 0.65 cd
Salinity treatments (S)	***	**	***	***	***	***	***	***	***	***
Genotypes (G)	NS	***	NS	***	***	***	***	***	NS	NS
G × S	*	**	NS	**	***	***	***	**	NS	NS

Values are means ± standard error of three replicates. Means followed by different letters in the same column are significantly different ($P < 0.05$, Duncan's multiple range tests).
Significance levels are expressed as: NS when $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

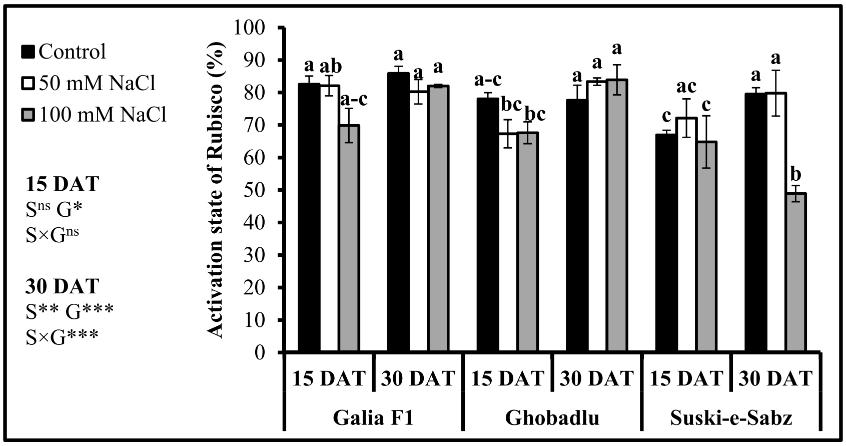

Fig. 1. Effect of NaCl treatments on activation state of Rubisco (%) in melon leaves at 15 and 30 DAT. Each column represents the average of three replicates (± SD). For a given sampling date, different letters indicate significant differences between salt treatments at $P < 0.05$.

Table 5
Summary of results of the stepwise multiple linear regression analysis for photosynthetic rate as dependent variable contributing to other traits as independent variables at 15 and 30 DAT under salinity stress.

15 DAT		30 DAT	
Independent Variable[a]	Parameter estimate	Independent Variable	Parameter estimate
Constant	−8.68**	Constant	−91.573**
E	5.2***	Fv/Fm	118.49**
WUE	1.356***	C_i	0.033**
C_i	0.016**		
g_s	−13.406*		
Rubisco total activity	0.099*		
R^2 (%)	99***	R^2 (%)	98***
Adjusted R^2 (%)	99	Adjusted R^2 (%)	97
Standard error of the estimate	0.102	Standard error of the estimate	0.375

The equation of the fitted model at 15 DAT is: Photosynthetic rate = −8.68 + 5.2 × transpiration rate + 1.356 × WUE + 0.016 × C_i - 13.406 × stomatal conductance + 0.099 × Rubisco total activity.
The equations associated with the fitted model at 30 DAT is: Photosynthetic rate = −91.573 + 118.49 × Fv/Fm + 0.033 × C_i.
Significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

[a] Only the variables with a significance of $P < 0.1$ were included for entry into the model. The variables were previously standardized.

with A_n in this study at 15 and 30 DAT were also presented in Table 5.

4. Discussion

In this study, we investigated the effects of salinity stress induced by 50 and 100 mM NaCl treatments on physiological and biochemical characteristics of two Iranian melon genotypes, "Ghobadlu" and "Suski-e-Sabz", as well as "Galia" F1 cultivar at 15 and 30 DAT, that was assessed by photosynthetic gas exchange parameters, chlorophyll fluorescence, Rubisco and NR activity, ABA content, and $\Delta^{13}C$. ANOVA results indicated that almost all of the measured traits were significantly affected by imposed stress condition at both sampling dates. Also, a notable reduction in biochemical components of carbon assimilation including Rubisco activity, chlorophyll content and Fv/Fm in parallel to g_s decline in studied melon genotypes suggests that specific negative effects of imposed saline stress on photosynthetic machinery could be attributed to the concurrent decrease of both stomatal and non-stomatal pathways.

4.1. Effect of salinity on water status and plant growth characteristics

RWC is a widely used measure of plant water status, in terms of evaluating the physiological impact of a cellular water deficit (Siddique et al., 2000; Farooq et al., 2009; Hassanvand et al., 2019). The ability of plants to maintain tissue hydrated during desiccation is an important strategy for resistance to stresses (Sánchez-Rodríguez et al., 2010). The relationship between RWC and the ability of plant genotypes to resist to drought and salinity was confirmed in previous studies, in which, the maintenance of high RWC was commonly considered as one of the best criteria to determine and classify the most sensitive and most tolerant tomato genotypes (Sánchez-Rodríguez et al., 2010; Aghaie et al., 2018) and melon landraces (Sarabi et al., 2017). Thus, a relatively high RWC (80–84%) that was recorded for both NaCl levels in melons at two different times might be considered as the relatively higher tolerance of genotypes studied under salt treatments.

Stomatal closure as one of the earliest responses to drought or osmotic stress conditions generated by high salinity, have a substantial role in water loss control in plants (Chaves et al., 2003). Accordingly, a strong positive relationship was observed between RWC and stomatal conductance at 15 and 30 DAT (r = 0.905, $P < 0.01$; r = 0.747, $P < 0.05$; respectively, Supplementary Table S1 and S2). This is in agreement with those observed in previous literature who stated that leaf water status interacts with the stomatal aperture (Lawlor and Cornic, 2002; Medrano et al., 2002).

In this study, osmotic potential decreased when salt treatments were applied. Such decreases were always greater at 100 mM NaCl and lower at 50 mM as compared with control treatments at both 15 and 30 DAT. At two different growth stages, "Ghobadlu" and "Galia" F1 had a better performance in terms of osmotic adjustment (lower osmotic potential), leading to a maintenance of higher RWC as compared with "Suski-e-Sabz", averaged over all saline treatments. Accordingly, with enhancement of salinity, leaf osmolality increases were significantly correlated with leaf RWC (r = 0.789, $P < 0.05$; r = 0.769, $P < 0.05$, Supplementary Tables S1 and S2). Salt accumulation in the medium culture causes progressively more negative water potential of the root zone. Therefore, plants must decrease their water potential in order to avoid cell dehydration and maintain a favorable gradient for water entrance from the medium into the root (Ruiz-Lozano et al., 2012).

In all melon plants, biomass significantly decreased in response to an increase of salinity in the nutrient solution. This result has also been reported in other studies on soybean (Bai et al., 2019), wheat (Elkelish et al., 2019) and maize (Hessini et al., 2019) under salt stress conditions. Reduced biomass under saline treatment could be attributed to the decreasing osmotic potential due to high concentration of sodium and chloride ions in the root zone (Greenway and Munns, 1980) leading to a nutritional imbalance (Pasternak, 1987). Carvajal et al. (1998) stated that osmotic regulation by electrolytes is likely the only way to coupling productivity with salt tolerance. In this regard, positive correlations were recorded between osmotic potential in melon leaves and biomass production at 15 and 30 DAT ($r = 0.728$, $P < 0.05$; $r = 0.659$, $P > 0.05$; respectively, Supplementary Tables S1 and S2) averaged over all salt treatments. These results are also in accordance with those reported by Puniran-Hartley et al. (2014) who stated that biosynthesis of compatible solutes (CS) is a very energy demanding process and increased osmotic pressure by CS accumulation in cell vacuoles comes at huge energy cost, which is often accompanied by a major decline in growth. Furthermore, many authors (Stępień and Kłobus, 2006; He et al., 2009; Ruiz-Lozano et al., 2012; Gong et al., 2013; Elkelish et al., 2019; Haider et al., 2019) concluded that lower accumulation of plant biomass under salt stress conditions is often directly due to photosynthesis inhibition. In accordance with these conclusions, we found strong positive correlations between biomass and A_n at two growth stages confirmed these statements ($r = 0.885$, $P < 0.01$; $r = 0.922$, $P < 0.01$; respectively, Supplementary Tables S1 and S2). Moreover, some studies have revealed that plants with higher salt tolerance exhibit less growth inhibition and had relatively higher growth rate and biomass production than salt sensitive ones (Singh and Sarkar, 2014; Chiconato et al., 2019). Accordingly, we found a higher capacity of "Galia" F1 and "Ghobadlu" than "Suski-e-Sabz" to sustain growth and production under salinity conditions.

At 15 and 30 DAT, the leaf area was positively correlated with the net photosynthetic rate ($r = 0.702$, $P < 0.05$; $r = 0.868$, $P < 0.01$; respectively, Supplementary Tables S1 and S2) and the biomass ($r = 0.686$, $P < 0.05$; $r = 0.742$, $P < 0.05$; respectively, Supplementary Tables S1 and S2). This suggests that salinity may also influence melon growth through a reduction in both photosynthetic rate and leaf area. Orsini et al. (2012) suggested that the maintenance of a functional leaf area may confer the most beneficial effects for cultivars prescribed to be used exclusively in marginal lands in terms of salinity and/or drought stress adaptation.

Our results indicated that leaves developed under salt conditions have a lower SLA than leaves produced under control conditions (Table 1). Similar results have also been reported in cowpea (Wilson et al., 2006), soybean (Bai et al., 2019) and *Phragmites karka* (Shoukat et al., 2019). Variations in SLA may be due to variations in leaf thickness and/or changes in density of leaf tissue (dry mass per unit volume) and it seems that an increase in biomass accumulation per unit of leaf area is a physiological characteristic in response to salinity treatment (Veneklaas et al., 2002; Wilson et al., 2006). As already stated, "Galia" F1 had the lowest SLA compared with two other genotypes studied at both sampling dates, indicating its better performance in terms of ability to accumulate more dry mass per unit of leaf area under salt stress.

4.2. Evaluation of DUALEX measurement to detect leaf chlorophyll and flavonol contents and N status under salinity treatments

Chl content measured with the DUALEX, as a leaf greenness index, decreased with increasing salt concentrations. This result is in accordance with our previous experiment, which revealed that salt stress decreased the chlorophyll content of melon genotypes and that the decrease depends on the salt tolerance of the plants (Sarabi et al., 2017). Accordingly, it may be concluded that "Galia" F1 was more tolerant than the two other genotypes in terms of having a higher chl at 15 and 30 DAT (i.e. higher investment of "Galia" F1 in photosynthetic apparatus per unit leaf area). The decrease in chlorophylls often occurs because of weakening and disintegration of pigment–protein–lipid complexes in chloroplast, increase in activity of chlorophyllase, and/or limitation of synthesis of proteins related with chlorophyll molecules (Levitt, 1980; Iyengar and Reddy, 1996; Jaleel et al., 2007; Moinuddin et al., 2016).

As shown in Table 2, a greater accumulation of flavonols was observed at 50 and 100 mM NaCl as compared with control, for both sampling dates. It has been reported that polyphenols (including flavonols) of leaves are stimulated when exposed to salinity stress (Ksouri et al., 2007; Hichem et al., 2009; Savirnata et al., 2010; Agati et al., 2011; Colla et al., 2013; Bibi et al., 2019). Bettaieb et al. (2011) stated that under stress conditions the accumulation of phenolic compounds is negatively related with plant biomass production. This was also the case in our study. A negative relationship between flavonols and biomass production was detected at both sampling times ($r = -0.590$, $P > 0.05$; $r = -0.647$, $P > 0.05$; respectively, Supplementary Tables S1 and S2). In this regard, Abreu and Mazzafera (2005) declared that under water deficit, secondary metabolites production increased via the reallocation of assimilated carbon, so that plant growth is reduced. In addition, under environmental stresses, the uptake of CO_2 is decreased due to stomatal closure leading to exposure of the chloroplast to an excess of excitation energy (Smirnof, 1993) and increased production of ROS (Li and Staden, 1988). In such conditions, epidermal phenolic compounds can function as an obstacle for the light to reach the mesophyll cells and thus reduce the chlorophyll excitation under water deficit imposed by salinity treatments (Nogués and Baker, 2000). Accordingly, it can be assumed that melons could tolerate the salt stress by stimulation of flavonoids accumulation in their leaves.

A high positive relationships were observed between NBI and NR at both sampling times ($r = 0.845$, $P < 0.01$; $r = 0.918$, $P < 0.01$; respectively, Supplementary Tables S1 and S2), which implies that NBI indicates nitrogen metabolism status in melon crops under salinity. Although this suggestion should be verified by assessment of other enzymes involved in nitrogen reduction and ammonium assimilation (e.g. nitrite reductase, glutamine synthetase, glutamate synthase, glutamate dehydrogenase) and their relations with NBI under saline condition.

In the current study, a positive relationship was observed between chl and A_n at both sampling times ($r = 0.575$, $P > 0.05$; $r = 0.898$, $P < 0.01$; respectively, Supplementary Tables S1 and S2), which indicated that the decline in chl contents may be considered a key physiological criteria for the lower A_n under salt stress in melons investigated. A similar relationship was reported in one sub-coastal species (*Paspalidium geminatum*) under NaCl stress (Moinuddin et al., 2016). This could be partly related to a nitrogen metabolism disturbance induced by stress (Gong et al., 2013) and as it was previously mentioned, the NBI and NR decreased significantly across all melons at both sampling dates ($P < 0.001$, Table 2). The most reasonable role of leaf nitrogen concentration in regulating A_n is the large fraction of this element in the photosynthetic apparatus e.g. chlorophyll molecules and Rubisco (Sage, 2013).

Significant inhibition of NR activity in response to increasing concentrations of NaCl in three genotypes implies that melon leaves could not sufficiently compartmentalize and storage a high concentration of Na and/or Cl in vacuoles, resulting thus in excessive accumulation of salt ions in the cytosol. Thus, NR (as a cytosolic enzyme) could be directly exposed to Na and Cl effects (Na and Cl concentrations in leaves were not measured in the present work). The NR activity depression induced by salt stress, could be an expression of stress induced enzyme degradation and/or metabolic inactivation, decline in gene expression and *de novo* synthesis of NR protein (Ferrario et al., 1998). Grattan and Grieve (1992) stated that decline in NR activity leads to a corresponding decrease in potential for nitrate reduction and its subsequent assimilation into protein and thus reduced plant growth. This is the case also in our study and a high positive correlation was observed between NR and biomass production at both sampling dates ($r = 0.743$, $P < 0.05$; $r = 0.914$, $P < 0.01$; respectively, Supplementary Tables S1 and S2 and Fig. 2).

It has been proved that nitrate reduction and amino acid synthesis in leaves needs a large input of ATP, redox equivalents (NAD(P)H) and carbon intermediates (Atkin et al., 2000). In leaves, these redox

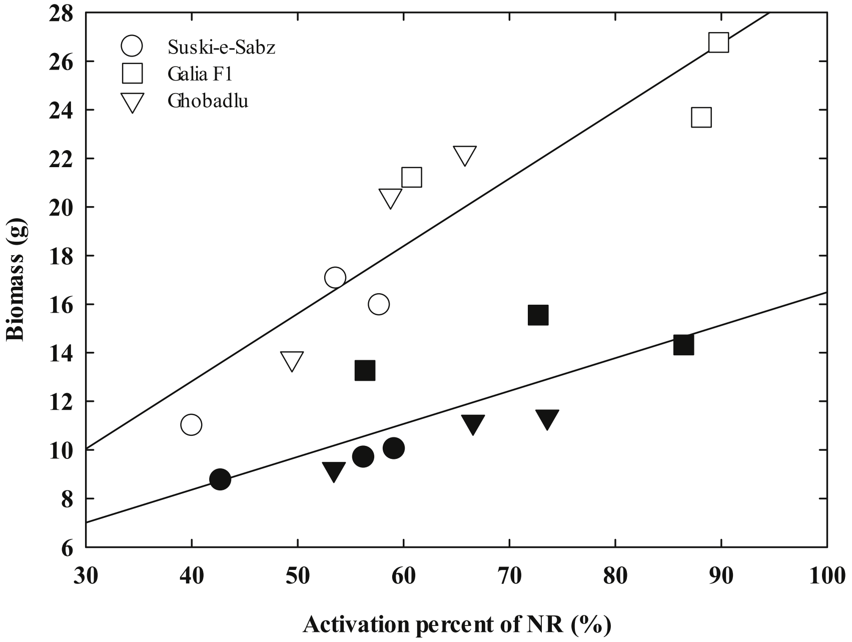

Fig. 2. Relationships between the activation percent of nitrate reductase (%) and biomass (g) of melon genotypes in the presence of different levels of salinity (0, 50 and 100 mM NaCl) at 15 DAT (closed symbols; $R^2 = 0.58$) and 30 DAT (open symbols; $R^2 = 0.85$). Data points are the mean values ($n = 3$).

equivalents can be obtained from several sources including photosynthesis (export of excess NADPH via the DHAP:3-PGA or OAA-malate shuttles) (Mann et al., 1978). Accordingly, highly positive relationships were observed between NR activity and A_n at 15 and 30 DAT ($r = 0.713$, $P < 0.05$; $r = 0.824$, $P < 0.01$; respectively, Supplementary Tables S1 and S2).

4.3. Role of abscisic acid

Some studies asserted that increased endogenous ABA content unexpectedly hinders plant growth (Dodd and Davies, 1996; Ryu and Cho, 2015). Accordingly, a negative relationship between ABA concentration and biomass was observed in plants studied at both sampling times ($r = -0.315$, $P > 0.05$; $r = -0.874$, $P < 0.01$, respectively, Supplementary Tables S1 and S2). In agreement with our results, the increase of ABA concentration under increasing salt stress caused general inhibition of growth in many other crops including Brassica species (He and Cramer, 1996), corn (Cramer and Quarrie, 2002), common bean (Cabot et al., 2009) and tomato (Babu et al., 2012).

In the current study, an increase in the leaf ABA concentration at 50 and 100 mM NaCl (with the exception of the 50 mM NaCl at 15 DAT in Suski-e-Sabz) caused a remarkable stomatal closure in all genotypes ($P < 0.01$, Table 3) and therefore, an inverse relationship between ABA and stomatal opening (g_s) observed at both sampling dates ($r = -0.437$, $P > 0.05$; $r = -0.591$, $P > 0.05$; respectively, Supplementary Tables S1and S2). Hence, it may be supposed that salinity effects on the biochemistry of photosynthesis are arising from ABA-induced stomatal closure. Stomatal closure in response to salinity-induced ABA increment has also been reported in tomato leaf tissues (Maggio et al., 2007; Lovelli et al., 2012).

ABA hormone signaling between root and shoot may have a critical role in regulating WUE (Brugnoli and Farquhar, 2000; Liu et al., 2019). Accordingly, both WUE and WUE_i were positively correlated with ABA concentration at 15 DAT, averaged over all genotypes ($r = 0.434$, $P > 0.05$; $r = 0.327$, $P > 0.05$; respectively, Supplementary Table S1). However, at the second sampling time, a negative relationship was observed between WUE and WUE_i with ABA ($r = -0.447$, $P > 0.05$; $r = -0.404$, $P > 0.05$; respectively, Supplementary Table S2). These contradictory results could arise from the fact that although the highest increase in ABA concentrations at 30 DAT was recorded in “Suski-e-Sabz” (7.7 fold at 100 mM NaCl with respect to control condition), this genotype displayed the highest stomatal conductance and transpiration rate in comparison with the two other genotypes, and consequently, the lowest WUE and WUE_i (Tables 2–4).

It has been demonstrated that ABA contributed to the activation of genes related to osmotic adjustment in plants under adverse conditions (Verslues and Zhu, 2005). In this regard, a negative correlation between ABA content and osmotic potential has been observed in genotypes studied at 15 and 30 DAT ($r = -0.435$, $P > 0.05$; $r = -0.608$, $P > 0.05$; respectively, Supplementary Tables S1 and S2). In addition, a study by Fricke et al. (2004) indicated that ABA was involved in an increase of xylem water potential and water uptake of plants under salinity stress.

Perales et al. (2005) using cell lines from two rice cultivars found that more salt tolerant lines had a higher ability for ABA biosynthesis than salt-sensitive ones. This result is not in accordance with our observation because “Galia” F1, a salt tolerant cultivar, showed a lower ABA accumulation as compared with the two other genotypes at 15 and 30 DAT. Notwithstanding, this cultivar had lower stomatal conductance and subsequently lower transpiration rate at 30 DAT (Table 3). Therefore, it can be assumed that “Galia” F1 may benefit from other physiological mechanisms to tolerate salinity condition rather than ABA accumulation.

4.4. Salinity responses of stomatal and non-stomatal components of photosynthesis

Our results indicate that the three melon genotypes respond to salt stress in a similar way by decreasing net CO_2 assimilation rate (A_n), stomatal conductance (g_s) and transpiration (E) with increasing level of salt stress. This decrease was accompanied by a significant reduction in C_i/C_a across all plants investigated and at both sampling dates.

Significant decrease in g_s has started at 50 mM NaCl for both sampling times when averaging all genotypes ($P < 0.001$, Table 3), which confirms previous reports that g_s is very sensitive to salinity stress (Netondo et al., 2004; Jiang et al., 2006a,b; He et al., 2009; Penella et al., 2015). This finding is not in agreement with some other data in the literature (e.g. Lu et al., 2003; Naumann et al., 2007) that reported stomatal aperture is resistant to salt stress. Diverse plant species or environmental conditions maybe caused these contradictory results (He et al., 2009).

Inhibition of net photosynthetic rate (A_n) caused by stomatal closure and down-regulating of Rubisco activity under imposed stress disrupts carbohydrate metabolism and thus reduces dry matter production (Chaves et al., 2002; Jurczyk et al., 2016). In the current study, strong positive correlations detected between biomass and A_n at both sampling periods ($r = 0.885$, $P < 0.01$; $r = 0.922$, $P < 0.01$; respectively, Supplementary Tables S1 and S2), proved that plant growth is tightly related to photosynthetic capacity (Sharma et al., 2005). Accordingly, the higher biomass production in “Galia” F1 and “Ghobadlu” with respect to “Suski-e-Sabz” seems to be related to a better ability of these genotypes in maintaining higher photosynthetic rates in response to salinity at 15 and 30 DAT ($P < 0.01$, Table 3). Similar results were also reported in melon and cucumber (Ruiz-Lozano et al., 2012) as well as sugarcane (Chiconato et al., 2019). A_n was also positively correlated with RWC in three genotypes ($r = 0.819$, $P < 0.01$; $r = 0.846$, $P < 0.01$; respectively, Supplementary Tables S1 and S2). This observation is in accordance with previous studies that reported photosynthetic rate of leaves is reduced as the RWC decreased (Lawlor and Cornic, 2002; Flexas et al., 2004; Guerfel et al., 2009; Huang et al., 2011). Hence, it could be concluded that the considerable decrease in stomatal conductance with increasing salinity at 15 and 30 DAT ($P < 0.001$) may help to keep tissue hydration (succulence) and also protect plants from a rapid dehydration (Orsini et al., 2012), consequently leading to maintained photosynthetic efficiency. In this experiment, although “Galia” F1 and “Ghobadlu” had a higher stomatal conductance and leaf transpiration rate at 15 DAT with respect to “Suski-e-Sabz”, they exhibited significantly lower g_s and E at 30 DAT, which were consistent with a better hydration state (higher RWC) and photosynthesis performance (i.e. higher WUE and WUE_i). This may have contributed to a better adaptation of these genotypes to imposed stress as growth stages progressed. In line with these findings, Maggio et al. (2006) have also asserted that increased salt tolerance is linked with low transpiration rates.

Salinity stress decreased A_n by 22% and 37%, g_s by 54% and 64% as well as E by 38% and 52% under 100 mM NaCl at 15 and 30 DAT, respectively. The greater decreases in g_s and E compared to A_n led to a 21–25% increase in the WUE and 42–44% in WUE_i at both sampling dates, respectively (Tables 3 and 4). Our data are in agreement with Moinuddin et al. (2016) who reported that transpiration (as a biophysical process) can be affected more than A_n (as a biophysical/biochemical process) by a decline in stomatal conductance, which leads to an increase in WUE, i.e. stomatal closure reduces water loss to a greater degree than CO_2 exchange and therefore WUE increases (Knight et al., 1994). This is consistent with our interpretation that the higher WUE can reflect higher photosynthetic capacity in studied melons. In addition, at first and second sampling dates, g_s ($r = 0.904$, $P < 0.01$; $r = 0.726$, $P < 0.05$; respectively, Supplementary Tables S1 and S2) was positively correlated with A_n. These relationships indicates some level of regulation between CO_2 demand by the chloroplasts and CO_2 supply by stomatal control (Farquhar et al., 1989).

As shown in Fig. 3, instantaneous carboxylation efficiency, as non-stomatal limitations to photosynthesis, estimated by the A_n/C_i ratio (Penella et al., 2015; Shoukat et al., 2019), decreased at 30 DAT averaging all genotypes under salinity condition ($P > 0.05$). Similar results have been reported for pepper accession, being used as rootstock (Penella et al., 2015), and physic nut (da Silva et al., 2011) exposed to salt stress, suggesting that salt stress has led to metabolic limitations to photosynthesis, probably in relation with diminished Rubisco carboxylase activity (da Silva et al., 2011), and/or decline in protein synthesis (Blevins, 1985; Bednarz et al., 1998). This is in accordance with obtained data on Rubisco activity in melon plants. In contrast, A_n/C_i significantly increased at 15 DAT under NaCl treatments ($P < 0.01$, Fig. 3). Therefore, it could be concluded that stomatal limitation had a greater impact on the suppression of photosynthetic rate at first sampling date.

The decrease in CO_2 fixation along with stomatal closure is often accompanied by depressed photosystem activity and electron transport rate (Bendaly et al., 2016). This condition caused over production of reactive oxygen species (ROS) by movement of electrons from photo excited PSII reaction center Chl (P680) to oxygen molecules rather than being used for carbon assimilation (Zribi et al., 2009), resulted in insufficient regeneration of both electron-accepting $NADP^+$ and ADP (Thapar et al., 2008; Else et al., 2009). Mateos-Naranjo et al. (2013) stated that decrease in net photosynthetic rate (A_n) could also be the result of salinity effects on the integrity or function of the photochemical apparatus (as one of the non-stomatal factors that limit photosynthesis). Accordingly, remarkable positive relationships were detected between Fv/Fm and A_n at 15 and 30 DAT in melon genotypes ($r = 0.687$, $P < 0.05$; $r = 0.957$, $P < 0.01$; respectively, Supplementary Tables S1 and S2). Similar results were also obtained in barley plants under saline treatments (Jiang et al., 2006a,b; Tavakkoli et al., 2011).

As previously mentioned the increase of the salt concentration was not connected with significant changes in the maximum quantum use efficiency of photosystem II (Fv/Fm) at 15 DAT ($P > 0.05$). This suggests that photoinhibition of PSII was not a major result of salinity stress in the short-term i.e., electron-transport processes appear not to be very salt-sensitive at first sampling date. However, Fv/Fm significantly decreased under salt stress at 30 DAT ($P < 0.01$), indicating photoinhibition and a diminished capacity for PSII electron transport (Stefanov et al., 2016). This decrease also demonstrated that the ability of PSII to reduce the primary acceptor QA was affected by salt concentrations (Krause, 1988). Some studies have shown that salt stress inhibits the PSII activity (Loreto et al., 2003; Akram and Ashraf, 2011; Shu et al., 2012, 2019, 2013; Gong et al., 2013; Cai et al., 2014; Moinuddin et al., 2016; Wani et al., 2019) whereas others have demonstrated no changes in the Fv/Fm measured under salinity (Dionisio-Sese and Tobita, 2000; Lu et al., 2002; Bendaly et al., 2016; Stefanov et al., 2016; Marriboina et al., 2017). These inconsistent results maybe a consequence of different sensitivities of the plant species and diverse experimental conditions (Stefanov et al., 2016). In this study, the highest Fv/Fm ratio was recorded in “Galia” F1 at 15 and 30 DAT. When averaged over salinity treatments this cultivar had a more efficient protection of photosystem II against photooxidative damage. Although some researchers reported that most salt-tolerant species maintain high Fv/Fm ratios under unfavorable conditions (Naidoo et al., 2008; Stepien and Johnson, 2009), lower Fv/Fm observed in “Ghobadlu” and “Suski-e-Sabz”, may be a means of down-regulating light harvesting and reaction center complexes which finally leads to reducing ROS production (as a salinity-tolerance strategy) (Moinuddin et al., 2016).

Rubisco assimilates CO_2 by the carboxylation of RuBP within the Calvin Cycle, and hence is the most obvious target in the photosynthetic capacity improvements of crops (Galmés et al., 2014). This enzyme is widely accepted as the major non-stomatal limiting step in photosynthetic carbon fixation (Jensen, 2000; Masumoto et al., 2005) and its activity is rapidly modulated during stress conditions (Field et al., 1998). In the current study, both the initial and total Rubisco activities were significantly reduced under NaCl stress. This may arise from reduced carboxylase activity of Rubisco, or by changes in the availability of either RuBP or CO_2 (Sharkey and Seemann, 1989). Moreover, it was stated that one of the factors that affect the activity of Rubisco under adverse condition is the genotypic difference of the cultivars (Arquero et al., 2006; Flexas et al., 2006; Zahoor et al., 2017) and, hence, “Suski-e-Sabz” exhibited a lower performance in terms of both initial and total activities as compared with two other genotypes.

The obtained results showed a strong positive relationships between photosynthesis (A_n) and Rubisco activities at both sampling times (Supplementary Tables S1 and S2). These results are also in agreement

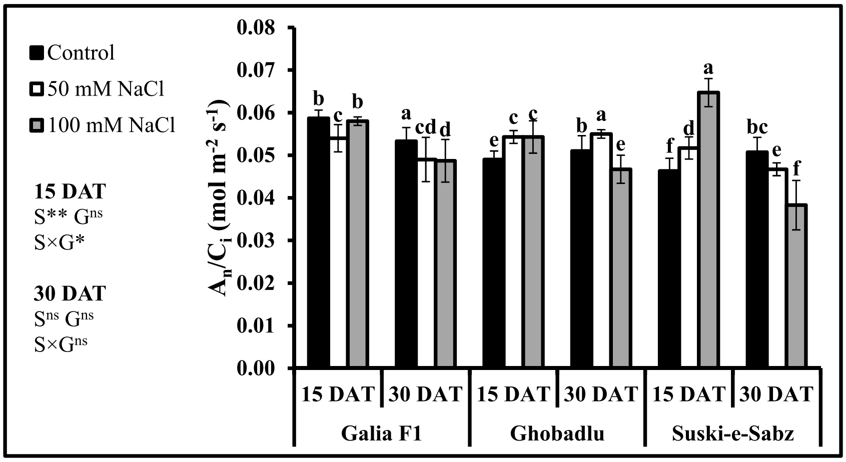

Fig. 3. Instantaneous carboxylation efficiency (A_n/C_i; mol m^{-2} s^{-1}) of melon genotypes (Galia F1, Ghobadlu, and Suski-e-Sabz) 15 and 30 days after exposure to NaCl treatments. Each column represents the mean values ± SE ($n = 3$). For a given sampling date, different letters indicate significant differences between salt treatments at $P < 0.05$.

with those observed in different plant species such as chick-pea (Soussi et al., 1998), melon (Centritto et al., 2000), cucumber (Huang et al., 2011) under salinity stress as well as cotton (Zahoor et al., 2017), tobacco and wheat (Parry et al., 2002) under drought. In addition, the significant biomass reduction under imposed stress supports the hypothesis that impeded carbohydrate synthesis may also be triggered by decreased Rubisco activity. This phenomenon could be associated with inhibited synthesis of Rubisco protein and/or Rubisco subunits (Jurczyk et al., 2016). In this regard, a reduction in soluble protein was observed in melon plants as salinity progressed (data not shown).

In the current study, initial and total activity of Rubisco were measured in order to assess whether imposed stress changed the activation state of the protein (Dann and Pell, 1989). This parameter displays the balance between the rate of deactivation and the rate of activase-promoted activation of Rubisco (Crafts-Brandner and Salvucci, 2000). As shown in Fig. 1, the general trend observed in the genotypes, with the exception of "Ghobadlu" at the second sampling time, was a decrease in activation state of Rubisco with increasing stress levels at 15 and 30 DAT ($P > 0.05$ and $P < 0.01$, respectively). Similarly to our result, the activation rate of Rubisco decreased in response to high temperature and CO_2 or low O_2 in cotton and tobacco (Crafts-Brandner and Salvucci, 2000).

4.5. *Using $\Delta^{13}C$ as an important proxy for indirect selection of melon genotypes under saline condition*

Because many variables such as heterogeneity of stomatal opening (i.e. ABA induced stomatal patchiness) (Beyschlag and Eckstein, 1998), time of measurement (Orsini et al., 2012), light conditions (Eisinger et al., 2000), changes in the cuticular conductance to vapour pressure (Boyer et al., 1997) caused non-uniform photosynthesis within leaves, application of instantaneous gas exchange measurements have some limitations and these effects must be considered either when these values are used as a means of effective screening of germplasm collections (Ashraf, 2004) or when these values are integrated into creating more accurate and complex models for predicting WUE (Orsini et al., 2012). Indeed, in many C_3 plant species, WUE and WUE_i have been shown to be negatively correlated to the discrimination against ^{13}C that occurs during CO_2 diffusion into leaf cells and photosynthesis (Eqn. (1)). Moreover, the ^{12}C and ^{13}C isotopes are stable and therefore the information inherent in the ratio of abundances of carbon isotopes, $^{13}C/^{12}C$, is invariant as long as carbon is not lost (Farquhar et al., 1989). So that $\Delta^{13}C$ can be used as a surrogate measure and a potential indicator of C_i/C_a and WUE, that produces data of high precision, which reflects the growing conditions over the entire measurement period.

As shown in Table 4, an increase in salinity resulted in a significant decrease in $\Delta^{13}C$ ($P < 0.001$), i.e. enrichment of ^{13}C in plant material under saline stress compared with control ones (3.7‰ and 3.5‰ lower discrimination against the heavier isotope in saline compared to non-saline treatments at 15 and 30 DAT, respectively). This observation confirmed previous reports of lower $\Delta^{13}C$ values following exposure to saline growing conditions in tissues of wheat (Shaheen and Hood-Nowotny, 2005; Yousfi et al., 2010), barley (Jiang et al., 2006a,b) and one sub-coastal species of marsh grasses (Moinuddin et al., 2016).

Since the carbon isotope values and the leaf RWC decreased at 15 and 30 DAT with increasing salinity, it can be suggested that the effect of salinity on $\Delta^{13}C$ values may in part be related to the water status of leaves. The positive correlation between $\Delta^{13}C$ and RWC at both sampling times confirmed this concept ($r = 0.814$, $P < 0.01$; $r = 0.835$, $P < 0.01$; respectively, Supplementary Tables S1 and S2). In addition, a high positive correlation between biomass and $\Delta^{13}C$ was observed ($r = 0.888$, $P < 0.01$; $r = 0.630$, $P > 0.05$; respectively, Supplementary Tables S1 and S2). The implication of this observation with melons is that $\Delta^{13}C$ could be a useful indicator when selecting these crops for biomass production under salinity conditions.

Rubisco catalyzes the initial step in the C_3 photosynthetic carbon reduction cycle, by adding CO_2 to RuBP to form two molecules of 3-phospho-D-glycerate (3-PGA). As it was previously stated in Eqn. (1), the isotope fractionation by Rubisco-catalyzed reactions is probably the main source of the large isotope discrimination shown by C_3 plants (kinetic effect) (Cleland, 1987; O'Leary, 1989; Cook, 1991). Accordingly, a high positive relationship was observed between both initial and total Rubisco activities with $\Delta^{13}C$ at both sampling dates (Supplementary Tables S1 and S2). Therefore, lower $\Delta^{13}C$ values recorded in plants under saline conditions may be arising from increased affinity of Rubisco in capturing the CO_2 delivered by stomatal and mesophyll conductance and eventually lowering C_i/C_a.

As expected from Eqn. (1), a significant positive relationship was observed between $\Delta^{13}C$ and C_i/C_a measured by gas exchanges in all genotypes ($r = 0.935$, $P < 0.01$; $r = 0.785$, $P < 0.05$; at 15 and 30 DAT, respectively, Supplementary Tables S1 and S2). Moreover, both $\Delta^{13}C$ and C_i/C_a of leaves decreased as melon plants were salt-stressed at both sampling dates. Bradford et at. (1983) observed that $\Delta^{13}C$ and C_i/C_a were greater in a tomato mutant lacking ABA than in its isogenic parent. This was the case at our first sampling date (15 DAT), since "Galia" F1 exhibited the lowest ABA concentration, and consequently it had the highest $\Delta^{13}C$ and C_i/C_a values as compared with two other genotypes. Conversely, at 30 DAT, this cultivar showed the lowest ABA concentration, but also had the lowest $\Delta^{13}C$ and C_i/C_a values. This implies that "Galia" F1 used other independent mechanisms (e.g. lower g_s and E) rather than ABA-dependent stress-signaling pathway, when plant has grown under salinity stress.

At the first sampling date, $\Delta^{13}C$ values were much higher than the theoretical values calculated based on Eqn. (1) (Fig. 4A). Therefore, it

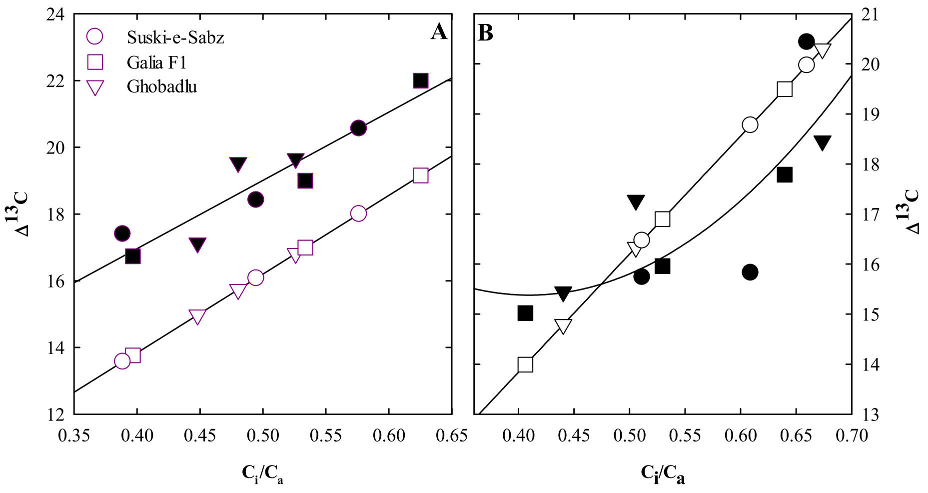

Fig. 4. Relationships between carbon isotope discrimination ($\Delta^{13}C$; ‰) and C_i/C_a ratio of melon genotypes in the presence of different levels of salinity (0, 50 and 100 mM NaCl) at 15 DAT (A) and 30 DAT (B). Closed symbols shows obtained $\Delta^{13}C$ from samples ($R^2 = 0.87$ and 0.65 at first and second sampling date; respectively), and open symbols shows estimated $\Delta^{13}C$ calculated based on Eqn. (1) with α = 4.4‰, and b = 28‰. Data points are the mean values ($n = 3$).

maybe suggested that most of structural carbon in melon genotypes studied at 15 DAT had been synthesized before any long-term effects of salinity, thus measured $\Delta^{13}C$ were higher than those predicted. The results of this experiment are in agreement with results in sugar beet cultivars under salinity stress (Dadkhah, 2013). However, at 30 DAT, generally, the measured $\Delta^{13}C$ values were lower than those estimated based on theory, with the exception of the "Galia" F1 at 100 mM NaCl, "Ghobadlu" at 50 and 100 mM NaCl as well as "Suski-e-Sabz" at control condition (Fig. 4B). Since the g_s was decreased to such an extent by the imposed salinity condition, only a small amount of carbon was available for new growth that resulted in reduced discrimination against the heavier carbon isotope at 30 DAT. Furthermore, the high scattering of data at the second sampling date might have partly contributed to the measurements of C_i/C_a for a short period of photosynthetic process, i.e. the values of C_i/C_a are not constant, while the $\Delta^{13}C$ values integrate carbon assimilation over the life of the plant material being analyzed (Condon et al., 2006; Kafi et al., 2007; Dadkhah, 2013).

$\Delta^{13}C$ was negatively correlated with WUE ($r = -0.868$, $P < 0.01$; $r = -0.632$, $P > 0.05$; Supplementary Tables S1 and S2) and WUE_i ($r = -0.845$, $P < 0.01$; $r = -0.645$, $P > 0.05$; Supplementary Tables S1 and S2; Fig. 5) at 15 and 30 DAT, respectively. Changes in water use efficiency can arise from either changes in stomatal aperture or assimilation capacity (both of which can affect C_i) (Matzner et al., 2001; Dadkhah, 2013). If stomatal conductance had the main effect on C_i, then the expected trend from the control condition to the highest salinity level would be an increase in WUE and decrease in C_i and $\Delta^{13}C$. If nevertheless, non-stomatal factors had the greatest influence on C_i, then the expectation would be a decrease in WUE and an increase in C_i and $\Delta^{13}C$ from the normal condition to highest stress (Dadkhah, 2013). In the current study, plants grown in the presence of salinity, at both sampling dates, had lower C_i and $\Delta^{13}C$ values and higher WUE, if compared with controls. Hence, it maybe stated that stomatal conductance had a greater effect than non-stomatal factors on C_i and consequently on $\Delta^{13}C$ and WUE. This assumption is in agreement with the results of Jiang et al. (2006a,b) who stated that reduced C_i for leaves grown under saline conditions has been ascribed to stomatal factors predominating over non-stomatal factors in the restriction of carbon assimilation. However, it is noteworthy that the balance between stomatal conductance and photosynthetic capacity, i.e., the balance between the supply and the demand functions for photosynthetic assimilation, determines the C_i/C_a (Farquhar and Sharkey, 1982). Therefore, a low value of C_i/C_a and correspondingly a smaller value of $\Delta^{13}C$ could have either resulted from low conductance or relatively high biochemical photosynthetic rate, or both, leading to

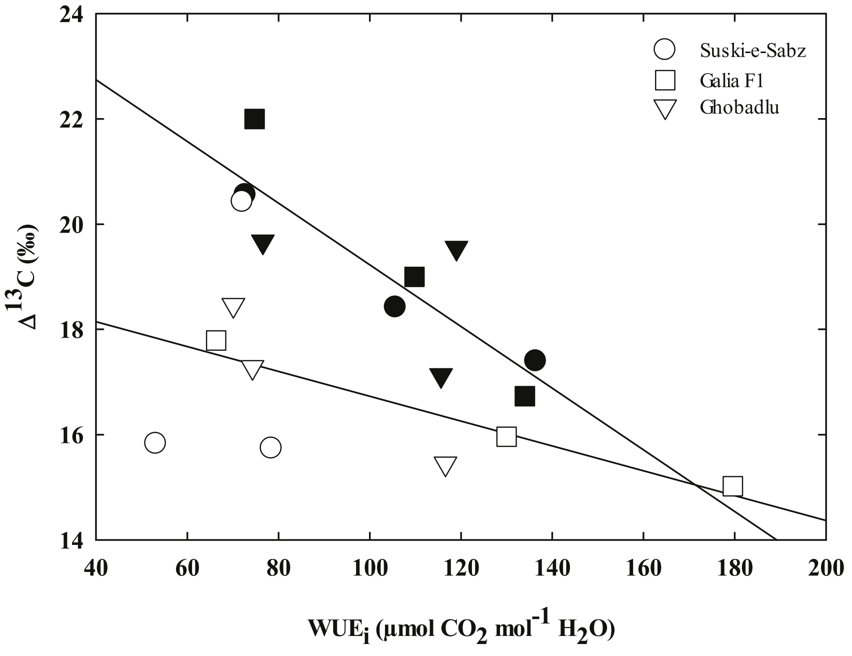

Fig. 5. Relationships between intrinsic water use efficiency (WUE_i; µmol CO_2 mol^{-1} H_2O) and carbon isotope discrimination ($\Delta^{13}C$; ‰) of melon genotypes in the presence of different levels of salinity (0, 50 and 100 mM NaCl) at 15 DAT (closed symbols; $R^2 = 0.72$) and 30 DAT (open symbols; $R^2 = 0.30$). Data points are the mean values ($n = 3$).

higher WUE (Sun et al., 1996; Brugnoli and Farquhar, 2000). Furthermore, Jiang et al. (2006a,b) reported that the magnitude of stomatal and/or non-stomatal limitations on photosynthesis rate is linked to the severity of imposed stress. Therefore, it has been proposed that under intermediate salinity, stomatal factors dominate the reduction in A_n, whereas non-stomatal limitations to A_n dominate at higher salt concentrations (Rivelli et al., 2002). According to above explanations, it seems that a simplistic approach to selecting of either stomatal or non-stomatal factors would be misleading.

4.6. Stepwise multiple linear regression

Based on multiple regression analysis, variations in photosynthesis rate under salinity stress at two sampling dates were mostly accounted by E, WUE, C_i, g_s, total activity of Rubisco and Fv/Fm, indicating that A_n could be estimated with high precision from the combination of these independent variables (standard errors of the estimations were 0.102 (μmol CO_2 m^{-2} s^{-1}) and 0.375 (μmol CO_2 m^{-2} s^{-1}) at 15 and 30 DAT, respectively, Table 5). In the other word, it may be concluded that both stomatal and non-stomatal factors are closely related to regulating the assimilation capacity of this crop under NaCl treatments.

5. Concluding remarks

Our results indicate that salinity caused a parallel decline in both stomatal and biochemical factors of the carbon assimilation process in melon and the analysis of plant carbon isotope discrimination ($\Delta^{13}C$) has contributed significantly to improving our understanding of the photosynthetic carbon metabolism under such stress.

$\Delta^{13}C$ is under strong genetic control, and therefore it is possible that using $\Delta^{13}C$ would be a useful adjunct to conventional breeding programs for improving the efficiency of melons in regions with salinity problems.

Concomitantly reduced NR and Rubisco activities across plants studied under salinity treatments demonstrate that both NR and Rubisco enzymes share a common pattern of regulation related to decreasing stomatal conductance (as a result of imposed stress).

Our finding that flavonol contents of melons increased under salinity, suggested the hypothesis that mechanisms involving the biosynthesis of these compounds were stimulated under elevated salt concentrations and proposed their protective role in this crop.

In general, “Ghobadlu” and “Suski-e-Sabz” genotypes represented a suitable performance as did “Galia” F1, in terms of most measured parameters e.g., RWC, LA, Ψ_S, flavonol content, Fv/Fm, $\Delta^{13}C$, biomass, A_n and NR, which are in good accordance with the genotypes origins and demonstrated their high adaptability to salinity stress. “Suski-e-Sabz” is cultivated in the center of Iran and at the margin of Dasht-e Kavir desert and the origin of “Ghobadlu” is in the North West of Iran and Eastern margin of Lake Urmia, the sixth largest saltwater lake on the Earth. Therefore, they could be put forward for future use in breeding programs, as promising salt-tolerant parents.

Authors’ contributions

The experiment was supervised by Jaleh Ghashghaie. Behrooz Sarabi, Jaleh Ghashghaie, Nasser Ghaderi, Sahebali Bolandnazar, Franz-Werner Badeck, Chantal Fresneau and Peter Streb conceived and designed the experiment. Behrooz Sarabi, Chantal Fresneau, Peter Streb, Sylvie Citerne, Maëva Tangama, and Andoniaina David helped in conducting the experiment. Behrooz Sarabi collected and analyzed the data and wrote the draft manuscript. Jaleh Ghashghaie, Nasser Ghaderi, Sahebali Bolandnazar, Franz-W. Badeck, Peter Streb and Chantal Fresneau revised and corrected the draft manuscript. All authors were involved in finalizing the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.05.010.

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