FRED AUGUSTO LOURÊDO DE BRITO

EFFECTS OF SALT STRESS ON GROWTH AND METABOLISM OF TOMATO (Solanum lycopersicum L.) PLANTS ASSOCIATED WITH HIGH CONCENTRATION OF CARBON DIOXIDE

Thesis presented to the Universidade Federal de Viçosa, as part of the requirements of the Graduate Program in Plant Physiology, to obtain the title of Doctor Scientiae.

Advisor: Dimas Mendes Ribeiro

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ABSTRACT

BRITO, Fred Augusto Lourêdo de, D.Sc, Universidade Federal de Viçosa, November, 2020. **Effects of salt stress on growth and metabolism of tomato (Solanum lycopersicum L.) plants associated with high concentration of carbon dioxide**. Advisor: Dimas Mendes Ribeiro. Co-advisor: Agustin Zsögön.

Soil salinity is an important environmental factor that limits the crop yield. On the other hand, the elevated CO_2 concentration (e[CO_2]) is able to mitigate the negative effects of salt stress on crop yield by stimulating photosynthetic rate in many C3 species, including Solanum lycopersicum. However, the impact of soil salinization on the relationship between biomass allocation, hormone biosynthesis and the primary metabolism of tomato plants under e[CO_2] are hitherto not well understood. In this context, tomato plants grown under salt stress showed high Na^+ concentration in tissues under both ambient [CO_2] (a[CO_2]) and e[CO_2]. Under a[CO_2], plants treated with NaCl showed lower accumulation of biomass compared to untreated plants. However, e[CO2] restored the growth of tomato plants under saline stress by reducing concentration of abscisic acid (ABA) and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid in leaves and roots. In addition, plants treated with NaCl under a[CO_2] showed reduction of the concentration of Krebs Cycle intermediates and increase of amino acids glycine and serine, while the plants under e[CO_2] treated with NaCl presented the recovery of these parameters to the levels of the control plants. These findings led to a new questioning whether plants with alterations in ABA biosynthesis present differential strategies of tolerance to saline stress under e[CO_2]. Thus, we analyzed tomato plants cv. Micro-Tom (MT), ABA-deficient mutant notabilis (not) and plants with high ABA concentration (NCED) submitted to salt stress. The growth of not plants was more affected in relation to MT and NCED plants, mainly under conditions of salt stress under both [CO_2]. On the other hand, e[CO_2] led to increases in total biomass and leaf area for all genotypes under saline stress, compared to a[CO_2]. In addition, NCED mutants showed greater growth in relation to the MT and not genotypes under e[CO_2] in control and saline conditions. e[CO_2] caused an increase in photosynthesis and reduction of photorespiration in the MT, not and NCED treated with NaCl compared to a[CO_2]. In addition, e[CO_2] induced changes in the primary metabolism which were associated with increases in dark respiration, especially of MT and not genotypes under saline stress. Taken together, our results suggest that e[CO_2] alleviates the effects of saline stress on plants through increased photosynthesis, reduced photorespiration and reprogrammed primary metabolism by mechanisms independent of ABA concentration.

Keywords: Photosynthesis. Respiration. Primary metabolism. Hormonal regulation. Salt stress. Tomato plant

RESUMO

BRITO, Fred Augusto Lourêdo de, D.Sc, Universidade Federal de Viçosa, novembro de 2020. **Efeitos do estresse salino no crescimento e metabolismo de plantas de tomate (Solanum lycopersicum L.) associados com a elevada concentração de dióxido de carbono**. Orientador: Dimas Mendes Ribeiro. Coorientador: Agustin Zsögön.

A salinidade do solo é um importante fator ambiental que limita a produtividade das culturas. Por outro lado, a elevada concentração de CO_2 (e[CO_2]) é capaz de mitigar os efeitos negativos do estresse salino no rendimento da produção, estimulando a taxa fotossintética em muitas espécies C3, incluindo Solanum lycopersicum. No entanto, o impacto da salinização do solo nas relações entre alocação de biomassa, biossíntese de hormônios e o metabolismo primário de plantas de tomate sob e[CO_2] até agora não são bem compreendidos. Nesse contexto, plantas de tomate crescendo sob estresse salino apresentaram elevada concentração de Na^+ nos tecidos em ambas concentrações ambiente (a[CO_2]) e e[CO_2]. Sob a[CO_2], plantas tratadas com NaCl mostraram menor acúmulo de biomassa em relação as plantas não tratadas. No entanto, e[CO_2] restaurou do crescimento de plantas de tomate sob estresse salino através da redução da concentração de ácido abscísico (ABA) e do ácido 1-aminociclopropano-1-carboxilico (ACC), precursor do etileno em folhas e raízes. Além disso, plantas tratadas com NaCl sob a[CO_2] apresentaram redução dos níveis de intermediários do ciclo de Krebs e aumento de aminoácidos glicina e serina, enquanto as plantas sob e[CO_2] tratadas com NaCl apresentaram recuperação desses parâmetros aos níveis de plantas controle. Esses achados levaram a um novo questionamento se as plantas com alterações na biossíntese de ABA apresentam estratégias de tolerância diferenciais ao estresse salino em e[CO_2]. Desse modo, nós analisamos plantas de tomate cv. Micro-Tom (MT), mutantes de tomate notabilis (not) deficientes em ABA e mutantes com alta concentração de ABA (NCED) submetidas a estresse salino. O crescimento de plantas not foi mais afetado em relação as plantas MT e NCED, principalmente em condições de estresse salino em ambas [CO_2]. Por outro lado, a e[CO_2] levou aumentos na biomassa total e área foliar para todos os genótipos sob estresse salino, em relação a a[CO_2]. Ademais, mutantes NCED apresentaram maior crescimento em relação aos genótipos MT e not em tratamentos controle e salino. A e[CO_2] promoveu aumento da fotossíntese e redução da fotorrespiração nos genótipos MT, not e NCED tratados com NaCl em comparação com a a[CO_2]. Além disso, a e[CO_2] induziu alterações no metabolismo primário as quais foram associadas com incrementos da respiração, especialmente nos genótipos MT e not sob estresse salino. Em conjunto, nossos resultados sugerem que a e[CO_2] alivia os efeitos do estresse salino nas plantas pelo aumento da fotossíntese, redução da fotorrespiração e reprogramação do metabolismo primário por mecanismos independentes da concentração de ABA.

Palavras-chave: Fotossíntese. Respiração. Metabolismo primário. Regulação Hormonal. Estresse salino. Tomateiro

SUMMARY

GENERAL INTRODUCTION

Crop exposure to salt stress under elevated CO_2: physiological, biochemical and molecular responses

Growth and yield of most crop plants are affected by saline soils, generating negative impacts in economic, environmental and social terms (Zörb et al., 2019). Salt accumulation in soils is a result of irrigation water that contains sodium chloride (NaCl), inadequate drainage of irrigated land and high temperatures (Minhas et al., 2020). High salt levels in soils lead to accumulation of Na^+ and Cl^- in the roots, reducing the ability of a plant to take up water (van Zelm et al., 2020). High concentrations of salt ions in plant tissues inhibit crop growth by impairing metabolic functions and photosynthetic carbon assimilation. In this context, elevated atmospheric CO_2 concentration could mitigate the negative effects of salinity on growth and crop yield by increasing photosynthetic rate in many C_3 species (Poorter and Pérez-Soba, 2001; Geissler et al., 2009; Piñero et al., 2016). On the other hand, rising CO_2 concentrations will be accompanied by increases in atmospheric temperature, which generally have a negative impact on crop yield (Ruiz-Vera et al., 2013). Moreover, elevated CO_2 concentration could reduce the nutritional quality of crops, through decreased mineral composition and increased caloric levels (Smith and Myers., 2018; Ebi and Loladze., 2019). Atmospheric CO_2 concentration currently sits at a record high level of 415 ppm and is expected to reach 685 ppm by 2050 (Tans and Keeling, 2020). Thus, a deeper understanding of how multiple environmental factors such as CO_2 concentration, temperature and salinity interact to regulate plant growth, yield and nutritional content is urgently required to ensure food security over the coming decades. In this chapter, we review how plant growth may be modulated in response to salinity and ambient CO_2 concentration. We also discuss how the combined effect of salinity and elevated CO_2 concentration regulates crop yield.

1 Salt stress and the regulation of crop development under ambient and elevated CO_2 concentration

Salt accumulation in arable soil causes osmotic stress and ion imbalance to roots, disrupting plant growth and metabolism (van Zelm et al., 2020). Moreover, plant responses to salinity stress are influenced by rising atmospheric CO_2 concentration (Figure 1). Root responses to salt stress include the production of reactive oxygen species (ROS), phospholipids and alteration of cytoplasmic free Ca^{2+}, which may transduce the salt stress signal to downstream protein activity and gene transcription (Bai et al., 2018). The resulting alteration of gene expression at the cellular level then influences metabolism and development of the whole plant in response to salt stress. Salt stress increases abscisic acid (ABA) concentration in plants by regulating the expression of 9-cis-EPOXYCAROTENOID DIOXYGENASE (NCED), a gene encoding the key enzyme for ABA biosynthesis (Wang et al., 2015; Silva et al., 2018). Interestingly, high CO_2 concentration alters the capacity for salt-induced ABA biosynthesis (Figure 1). In tomato, the largest horticultural crop in the world, atmospheric CO_2 enrichment decreased ABA and also ethylene production by roots, leading to maintenance of metabolic processes and sustained root growth under salt stress conditions (Brito et al., 2020). Moreover, tomato plants grown under elevated CO_2 concentration tolerate higher root-zone salinity levels than plants grown under ambient CO_2 concentration (Maggio et al., 2002). Whether the production of ABA and ethylene was responsible for tolerance of tomato plants to salt stress under high CO_2 remains to be determined. In addition to the changes in hormone levels, NaCl treatment resulted in lower mineral uptake and translocation in roots of barley (Hordeum vulgare L.) grown under ambient CO_2, which were restored by elevated CO_2 concentration, also salt stress combined with high CO_2 concentration leads to increased carbon allocation to roots, contributing to nutrient uptake (Pérez-López et al., 2014). Furthermore, the greater sugar allocation from source leaves to roots under elevated CO_2 could stimulate enzymatic and metabolic antioxidative defenses in roots, allowing ROS homeostasis during salt stress (Keunen et al., 2013). Taken together, these studies demonstrated that there is a close interplay between salt stress, carbon availability and root development. However, there is a need for a deep understanding on the molecular mechanisms by which salt stress and high CO_2 modify root development.

Plant shoots exhibit many physiological responses to high salt and CO_2 concentration (Figure 1). The mitigating effect of elevated CO_2 concentration on growth of many species of crop plants suffering from salt stress is attributed to increased photosynthetic efficiency (Poorter and Pérez-Soba., 2001, Geissler et al., 2009). For instance, the net photosynthetic rates of musk melon (Cucumis melo) plants upon treatment with 50 mM NaCl were found to be 75% greater under 800 ppm CO_2 than in 400 ppm CO_2 (Mavrogianopoulos et al., 1999). Although salt stress still inhibited photosynthetic carbon fixation in barley under high CO_2, a remarkable increase in the maximal rate of net photosynthesis was observed in plants treated with 80, 160 and 240 mM NaCl grown at 700 ppm CO_2 (Pérez-López et al., 2012). Moreover, tomato plants grown at 400 ppm CO_2 in soil supplemented with 150 mM NaCl showed a 17% reduction in net CO_2

assimilation rate compared to untreated control plants, while at 750 ppm CO_2 photosynthesis was not reduced in the presence of salt stress (Brito et al., 2020). The increased net CO_2 assimilation rate described above was accompanied by an increase in shoot growth rate. Taken together, it appears that an increased CO_2 supply for photosynthesis is essential to maintain plant growth under salt stress conditions.

Salt stress increases the concentration of ROS and thus inhibits the development of root and shoot by impairing enzymatic, cell wall and membrane functions (Huang et al., 2019). In tomato, elevated CO_2 concentration induced the expression of RESPIRATORY BURST OXIDASE 1 (SlRBOH1), which regulates tolerance to oxidative stress (Yi et al., 2015). The increased expression of SlRBOH1 reduced leaf transpiration, decreased root-to-shoot Na^+ transport and subsequently inhibited the negative effect of salt stress on photosynthesis and biomass production in tomato plants under elevated CO_2 concentration (Yi et al., 2015). Salt stress also increased the concentration of ABA and ethylene in shoots of tomato plants (Figure 1); however, high CO_2 concentration resulted in a lower concentration of these hormones in leaves, which allows for plant growth even under soil salinity (Brito et al., 2020). In addition, high CO_2 alleviates the effect of salinity in tomato plants by maintaining the metabolic processes that, in turn, increase rates of photosynthetic carbon assimilation as compared to plants under ambient CO_2 concentration (Brito et al., 2020). Efforts to enhance yield of crops grown under salt stress conditions must, therefore, be accompanied by an improved understanding of the contribution of ROS and hormone signaling to the coordination of carbon metabolism and growth in response to increasing atmospheric CO_2 concentration.

2 Salt-tolerance of crops in response to high CO_2 and modifying factors

Crop plant responses to salinity are influenced by several factors, including the severity, duration and rate at which salt stress is imposed (Figure 2). Hence, excess concentration of several salts present in the rhyzosphere may cause a substantial reduction in crop yield. For instance, muskmelon productivity was affected by increasing NaCl concentration of irrigation water. On the other hand, whereas yield of muskmelon plants was strongly increased when elevated CO_2 concentration was added to plants treated with 25 mM NaCl, there was only a slight stimulation of yield by high CO_2 at 50 mM NaCl (Mayrogianopoulos et al., 1999). Moreover, increasing CO_2 concentration leads to enhanced growth of most plants in situations of mild to moderate salt stress, while at high salt concentrations the effects of high CO_2 on growth are marginal (Poorter and Perez-Soba., 2001). Nevertheless, salt concentration in the soil is highly dynamic, with warm temperatures and evaporation/transpiration of soil water, soluble salts move to the upper soil layers and generate increasing salt stress in the active root zone (Nachshon., 2018). Therefore, in agricultural environments, plants are usually exposed to gradually increasing salt concentrations. The common practice of using saline groundwater in irrigated agriculture drives faster soil salinization in regions faced with water scarcity, as the rate at which salt accumulates in the rootzone may exert a differential response on plant metabolism. This can be illustrated by the tomato plants exposure to gradual or acute increasing concentration of NaCl under elevated CO_2 concentration. It was reported that high CO_2 (760 ppm) decreased stomatal conductance (g_s) of tomato plants treated with 100 mM NaCl, when salt was applied in a single initial step in the nutrient solution (Yi et al., 2015). This observation was accompanied by a decrease in root-to-shoot Na^+ transport, correlating with the improvement in salt tolerance (Yi et al., 2015). A different response occurs under conditions of gradual application of NaCl, 50 mM increments of NaCl every second day, until a predetermined salt concentration is reached. In this way, when 150 mM NaCl was applied gradually to tomato plants, elevated CO_2 (750 ppm) kept the gs of salt-treated plants similar to the values observed in plants grown without salt stress under ambient CO_2 concentration (Brito et al., 2020). As a result, the gradual application of NaCl did not alter Na^+ levels in leaves of tomato plants growth under both CO_2 concentrations. In the same study, the reason for restoration of tomato plant growth under salt stress in high CO_2 was a consistent maintenance of primary metabolism in combination with reduced biosynthesis of ABA and ethylene. These observations highlight the flexibility of plants' response to the rate at which salt stress is imposed and imply that salt stressed-plants are able to adjust their growth patterns in distinct ways to elevated CO_2 concentration.

Tolerance to salt stress varies according to species, genotype and developmental age of the plant (Figure 2). Tolerance of different plants to salt stress varies substantially across crop species and, to a lesser extent, between genotypes (Minhas et al., 2020). This becomes clear when looking at the effects of salinity on tomato plants, modern tomato cultivars are sensitive to moderate levels of salt stress, but there is considerable variation in the salt tolerance in wild tomato species such as S. galapagense, S. cheesmaniae, S. chmielewskii, S. habrochaites, S. lycopersicoides, S. pennellii and S. pimpinellifolium (Li et al., 2011; Pailles et al., 2020). For instance, salt-tolerant accessions of S. cheesmaniae respond to Na^+ accumulation by producing more leaves than non-tolerant accessions. S. galapagense tolerance, on the other hand, is characterized by increased leaf succulence (Pailles et al., 2020). Additionally, mechanisms of tolerance to salt stress in tomato plants can also be found in structural characteristics, cellular changes, responsive genes and signaling pathways.

To develop a salt tolerant cultivar in a future with rising atmospheric CO_2 concentration, more knowledge is needed on the integrative effects of salt tolerant mechanisms and high CO_2. In this context, leaf gas exchange and water relations are of special importance because CO_2 mainly influences salinity tolerance mechanisms associated to the osmotic effect of salinity rather than to ion specific effects. The effects of salt stress on crop plants growth are also dependent on the plants' developmental age. In maize (Zea mays), salinity response of growing leaf cells was linked to cell age, since the size of younger cells increased upon salt stress through the increased protection from ROS, while intercellular accumulation of ROS decreased growth of older cells during leaf growth (Kravchik et al., 2013). In general, crop plants at early growth stages are more sensitive to increases in soil salinity than plants at later stage, which could have a negative impact on crop yield (Arzani et al., 2008; van Zelm et al 2020). Interestingly, elevated CO_2 concentration also has effects on plant development at early stages. Thus, the response to CO_2 is age-dependent, as the plants have a limited window of responsivity for acclimation. Gasparini et al. (2019) found that elevated CO_2 could increase growth of tomato plants deficient in gibberellins synthesis, but this effect varied dramatically depending on the stage of plant development at which CO_2 was applied. The extent to which elevated CO_2 concentration contributes to growth regulation of crop plants during development under salinity conditions still needs to be explored.

Salt tolerance mechanisms and their interaction with elevated CO_2 concentration is not straightforward, because fluctuations in temperature and light intensity influence plant response to salinity under high CO_2 (Figure 2). For instance, high CO_2 maintains homeostasis of antioxidant system and carbon metabolism of tomato plants under salt stress grown in environment with fluctuations in irradiance and temperature (Brito et al., 2020). On the other hand, tomato plants exposed to constant irradiance and temperature during the day may encounter disturbances in homeostasis of antioxidant system and activate general responses to salt stress under elevated CO_2 concentration, thus, stress tolerance mechanisms could represent additional costs to the plants (Munns and Gilliham., 2015; Yi et al., 2015). In addition to these alterations on plant metabolism, evidence shows that phenotypes of plant grown in controlled conditions under constant irradiance and temperature are significantly different from plants of the same genotype grown in natural environments (Mishra et al., 2012; Bruce et al., 2015). Screening experiments focusing on plant performance under salt stress often involve the introduction of the candidate plants in hydroponic systems or in soil containing high salt concentration in controlled environment chambers under constant irradiance and temperature (Bado et al., 2016). Since the results obtained from plants grown in controlled environments might not be representative of natural conditions, plant growth in more natural conditions seems to be crucial in crop breeding for salinity tolerance (Poorter et al., 2016; Annunziata et al., 2017).

3 Mechanisms of crop tolerance to salt stress in response to elevated CO_2

3.1 Regulation of photosynthetic carbon metabolism

Given its close similarity to the K^+ cation, Na^+ enters the shoot symplast from the xylem apoplast passively via nonselective cation channels (NSCC), and subsequently moves into mesophyll cells (Figure 3). The Na^+-permeable aquaporins in the plasma membrane of bundle sheath cells represent a likely entry point of Na^+ into the cell. Hyperosmotic stress caused by salinity increases the concentration of ABA, ethylene and H_2O_2, and reduces K^+ availability in shoots (Hedrich et al., 2018). As a result, stomata close, reducing gas exchange, growth and crop yield. Elevated CO_2 concentration also leads to reduced stomatal conductance, however, plant growth is maintained or increased (Ainsworth and Rogers., 2007). This can be explained by variation in Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) carboxylation efficiency under high CO_2 (Figure 3). Elevated CO_2 concentration increased the photosynthetic rate in soybean (Glycine max) by 20% over the course of the growing season in the field (Bernacchi et al., 2005). High CO_2 resulted in a decrease in the concentration of both ABA and 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene, in tomato leaves of plants grown under high salinity, which led to an increase in stomatal aperture when compared to plants treated with NaCl at ambient CO_2 (Brito et al., 2020). This suggests that leaves under high CO_2 are poised to generate hormone signals more slowly during salt stress (Figure 3). Consequently, the hormone sensitivity of guard cells should differ between ambient and elevated CO_2 concentration. In this context, fine-tuning of guard cell activity of plants grown under elevated CO_2 concentration could increase yield under salinity conditions, the result of an increased ability for CO_2 assimilation. In fact, atmospheric CO_2 enrichment leads to a higher rate of photosynthesis for many C3 species under salt stress (Poorter and Pérez-Soba., 2001; Geissler et al., 2009; Piñero et al., 2016). In the Calvin cycle, RubisCO catalyzes the addition of CO_2 to ribulose-1,5-bisphosphate (RuBP) to generate two molecules of glyceraldehyde-3-phosphate, which are then used to either regenerate RuBP or to synthesize sucrose, starch and amino acids (Stitt et al., 2010). Increased photosynthesis in salt-stressed plants under elevated

CO_2 may be due to increased intercellular CO_2 concentration, in line with a partial increase in stomatal aperture resulting from factors such as low concentration of ABA and ethylene (Brito et al., 2020). Salt stress also affects chloroplast ultrastructure and mesophyll conductance to CO_2 (g_m), indicating that modification of leaf structural characteristics could reduce plant salinity tolerance (Chaves et al., 2009; Geissler et al., 2009). High CO_2 seemed to mitigate the negative effects of salinity on photosynthesis. Therefore, the diffusion limitation through the stomata and the mesophyll, along with the alterations in photosynthetic metabolism, is tightly regulated by salt stress, and elevated CO_2 induces specific alterations to allow growth under salinity conditions.

Increased soil salt concentration decreases the Calvin cycle activity, which results in reduced energy production and $NADP^+$ regeneration, leading to over-reduction of the photosynthetic electron transport chain and to ROS production. Moreover, mitochondrial respiration is perturbed in plants under salt stress, which also increases ROS production in respiratory electron transport (Choudhury et al., 2017). Salinity increases photorespiration, leading to the production of H_2O_2 by glycolate oxidase in the peroxisome (Figure 3). Thus, salt stress that limits CO_2 availability due to stomatal closure enhances the production of ROS in shoot tissues that, in turn, can cause oxidative damage to proteins, membranes, RNA and DNA molecules. The ROS detoxifying proteins such as superoxide dismutase, ascorbate peroxidase, catalase, glutathione peroxidase and peroxiredoxin, as well as antioxidants present in subcellular compartments, play a key role in mitigating the oxidative process in cells and in preventing the harmful effects of salt stress (Hasanuzzaman et al., 2019). Genes traditionally associated with the response to ROS detoxification have been cloned from SR3 wheat, a hybrid with a high tolerance to salt stress (Dong et al., 2013). In this context, the importance of these pathways to prevent ROS toxicity is illustrated by the finding that overexpression of genes involved in ROS scavenging resulted in the maintenance of photosynthetic energy capture and yield improvement under salt stress (Siddiqui et al., 2017). Interestingly, many of these transgenic lines have reduced growth under non-saline conditions. One reason for this may be the energetic costs associated with ROS detoxification mechanisms (Munns et al., 2020). Since a higher CO_2 concentration maintains sufficient energy reserves in the cells (Figure 3), it can be considered as a positive factor for future crop productivity. Moreover, the ability of elevated CO_2 concentration to adjust patterns of assimilation, storage and utilization of carbon in response to salt stress may determine the reproductive success of crops.

The growth response to salt stress under ambient CO_2 concentration indicates that increases in both photorespiration and the oxygenase reaction of RubisCO leads to decreased biosynthesis of sucrose and starch, thereby reducing plant growth (Figure 3). By contrast, elevated CO_2 concentration stimulates carbohydrate biosynthesis because photosynthesis is CO_2-limited in the vast majority of plants under ambient CO_2 concentration (~415 ppm). In this context, plant growth is dictated by the rate of metabolites exported from the Calvin cycle, hence, suboptimal usage of carbon reserves would affect crop yield. On the other hand, if carbon export is increased in leaves under elevated CO_2 concentration, then the limitation of biosynthesis by carbon supply may be alleviated in plants under salt stress. Takagi et al. (2008) found that photosynthetic capacity and transport of carbon assimilates were increased in tomato plants treated with NaCl under elevated CO_2 concentration, in line with an increase in sink capacity resulting from factors such as decreased carbon accumulation in leaves and limited root growth. Thus, increased crop yield under salt stress can only be achieved when a plant has sufficient capacity to use the increased supply of carbon available at high CO_2, and this can be limited by the availability of minerals. Therefore, efficient nutrient acquisition is of prime importance for salinity tolerance. Mineral nutrition experiments revealed that high CO_2 caused adjustments in root size to maintain the uptake and transport efficiency of nutrients in barley plants under saline conditions (Perez-Lopez et al., 2014). The tonoplast-localized Na^+/H^+ exchanger (NHX) regulates Na^+ detoxification via sequestration of Na^+ within the vacuole (Apse et al., 1999). Interestingly, overexpression of the NHX1 in tomato increases root-to-shoot K^+ transport, which improves plant salt tolerance due to the increased intercellular K^+/Na^+ ratios (Leidi et al., 2010). In halophytes, reduction in K^+ efflux is associated with decreased H^+ efflux, which is an energy-saving strategy, allowing the distribution of more resources for plant growth (Percey et al., 2016). The most salt tolerant accessions of S. cheesmaniae and S. galapagense show high K^+ levels in their roots, providing strong evidence of the relation between K^+ uptake and tolerance to salt stress (Pailles et al., 2020). Together, these observations indicate that the adjustment of growth to salt stress conditions is not only dynamic but also highly flexible at elevated CO_2 concentration.

3.2 Functional role of carbohydrate metabolism in adaptation of roots to salt stress in response to high CO_2

Most of the research into the role high CO_2 plays on salinity tolerance has shown that the increase in the rate of carbon assimilation in crop plants was accompanied by an increase in root biomass (Brito et al., 2020; Garcia-Sanchez and Syvertsen., 2006; Pérez-López et al., 2014; Syvertsen and Levy, 2005). These results indicate that a supply of sugars from shoots is essential for the maintenance of root growth under saline conditions. In this context, sucrosederived glucose activates the protein kinase Target of Rapamycin (TOR) to control nutrient status and energy signaling and promote cell proliferation in the root meristem (Figure 4). Soil salinization inhibits primary root elongation by inhibiting cell proliferation in the root meristem (Liu et al., 2015). On the other hand, TOR modulates root meristem activity during salt stress (Fu et al., 2020). Since elevated CO_2 can result in higher levels of carbon in crop plants, efforts to improve crop performance in saline soil must be accompanied by an increased understanding of how the additional photosynthate will be used to control TOR signaling not only in roots but in organs such as flowers, fruits and seeds, which is core to agricultural productivity. Glucose also stimulates lateral root formation through interaction with brassinosteroids (Gupta et al., 2015). There is evidence that the lateral root formation is promoted when Na^+ level is low in the soil but inhibited when Na^+ levels are high (Ji et al., 2013). High CO_2 concentration can influence root system architecture by increasing carbon allocation to the roots (Freixes et al., 2002; Butterly et al., 2015). Thus, carbohydrate levels, together with hormones may be crucial for salt-regulated lateral root formation dynamics.

Salt stress induces biosynthesis of ABA and ethylene in tomato roots, which leads to root growth inhibition in response to ambient CO_2 concentration (Brito et al., 2020). Ethylene inhibits root cell elongation and cell proliferation by modulating auxin level, distribution and signaling (Mendez-Bravo et al., 2019). It has also been shown that auxin acts downstream of ethylene in mediating ABA-induced root inhibition (Qin et al., 2019). High CO_2 confers salt tolerance in tomato plants by decreasing the biosynthesis of ABA and ethylene in roots. The decrease in root accumulation of ABA and ethylene led to maintenance of carbon metabolism in roots, increased root growth and subsequently enhanced salinity tolerance (Brito et al., 2020). Nutrient uptake is closely linked to carbon availability in roots as sugars regulate the expression of genes that encode transporters or channels for ions such as NH^{4+}, SO_4^{2-}, K^+ and PO_4^{3-} (Lejay et al., 2003). Moreover, salinity increases ROS production in roots of crops and thereby restricts cell proliferation and cell elongation (Zhang et al., 2016). On the other hand, sugars stimulate enzymatic and metabolic antioxidative defense system in roots (Keunen et al., 2013). Thus, it is plausible that root sensitivity to ROS will differ between plants grown under ambient and elevated CO_2 concentration, as a result of their differences in carbohydrate levels. Therefore, the participation of sugars in ion uptake and antioxidant defenses might be an important salt tolerance strategy. However, how carbon acquired through photosynthesis coordinates the effects of sugar sensing in roots is hitherto not well understood.

4 Future prospects

Research into growth regulatory processes, under influence of salinity and in interaction with increasing atmospheric CO_2 concentration, indicates that high CO_2 increases the photosynthetic carbon gain of crops, mitigates oxidative stress, and can thus enhance yield in saline soils. The challenge is to understand how the increased amounts of carbohydrates are integrated with the rest of plant metabolism under salt stress conditions. Therefore, efforts to improve crop yield in saline soils must be accompanied by knowledge of signaling pathways at the core of resource sensing, carbon allocation, source-sink relations, and plant development. Growth measurements and molecular analyses of regulatory processes triggered by salt stress under CO_2 enrichment will need to consider the natural fluctuations in irradiance and temperature as well as the severity of salt stress. A deeper understanding of the physiological basis for salinity tolerance and the response to increased CO_2 could also inform biotechnological approaches to manipulate wild relatives of crops and accelerate crop breeding to meet future challenges using state-of-the-art gene editing technologies (Zsögön et al., 2017; Zsögön et al., 2018). Thus, both molecular breeding and advanced biotechnology methods should help researchers understand the effects of salinity on metabolism and growth of crop plants under future CO_2 concentrations.

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Figures

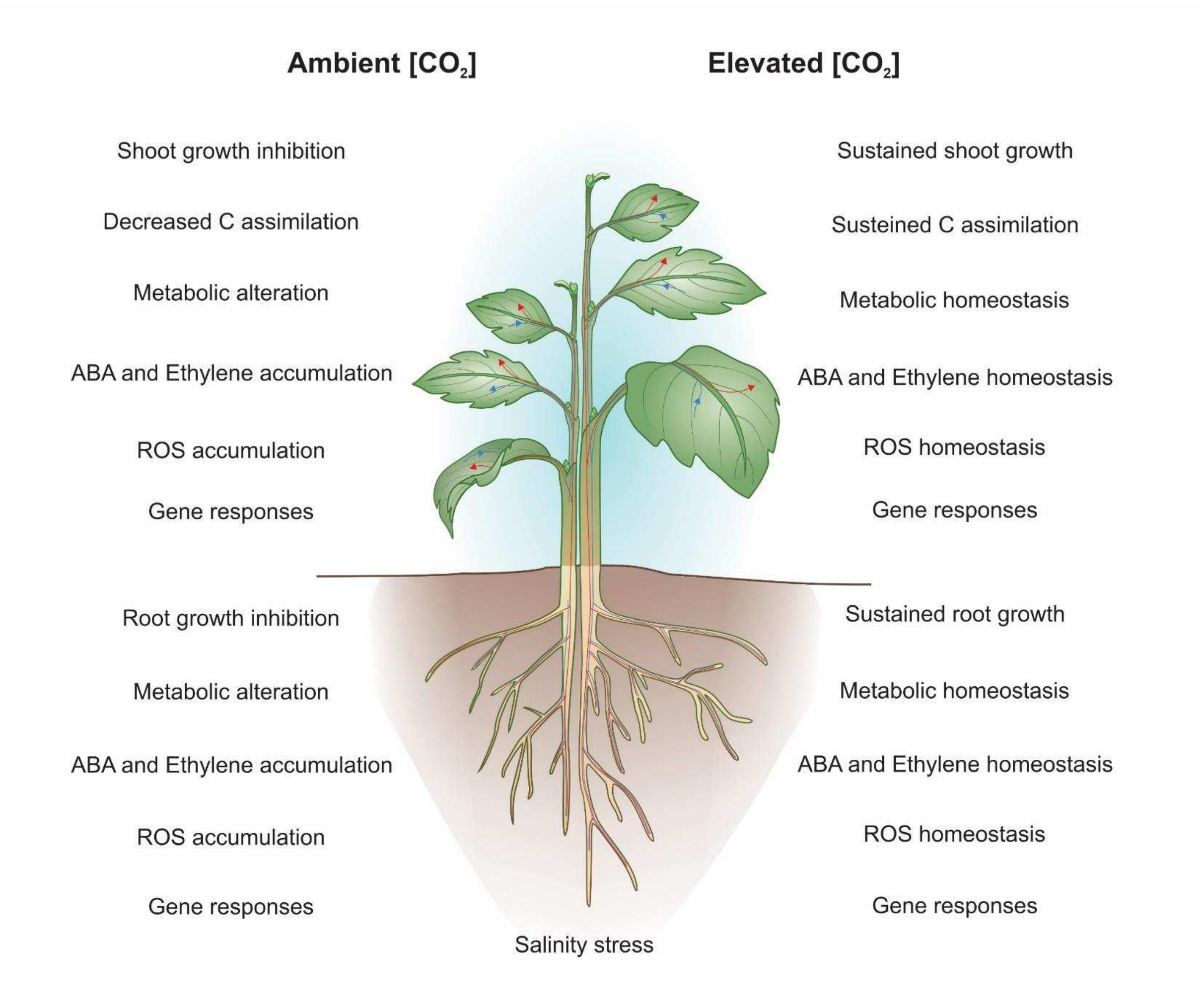

Figure 1. Salinity action and crop responses to increased CO_2 concentration. Salt stress triggers a wide range of plant responses, from alterations in gene expression and cellular metabolism to changes in growth and yield. The atmospheric CO_2 concentration influences how a crop plant responds to salt stress. Overall, plants show a salt-sensitive phenotype under ambient CO_2 concentration, whereas elevated CO_2 improves the performance of crops under salt stress.

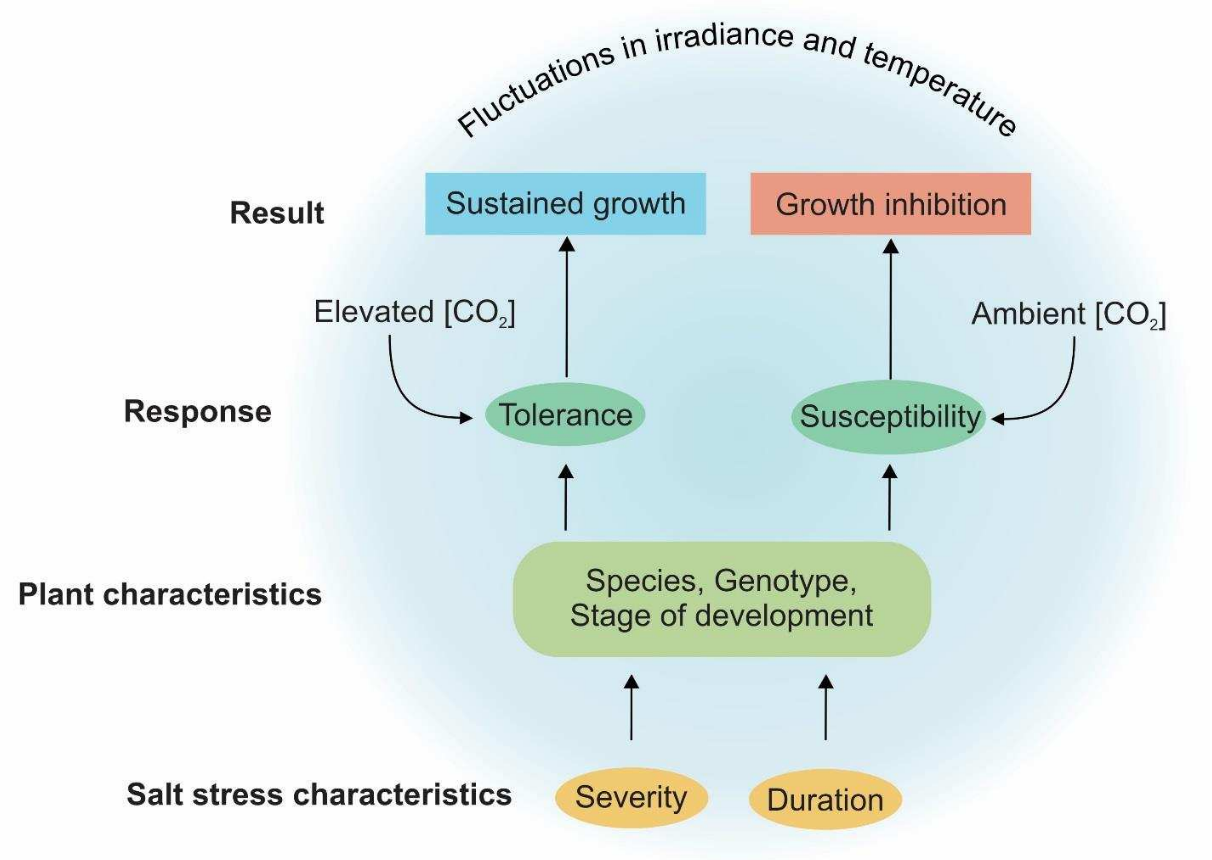

Figure 2. Physiological responses of crops to salt stress are determined by several factors. Plants respond to salt stress depends on severity, duration of exposure, age, species and genotype. Moreover, tolerance and susceptibility to salinity vary according to the atmospheric CO_2 concentration and fluctuations in irradiance and temperature.

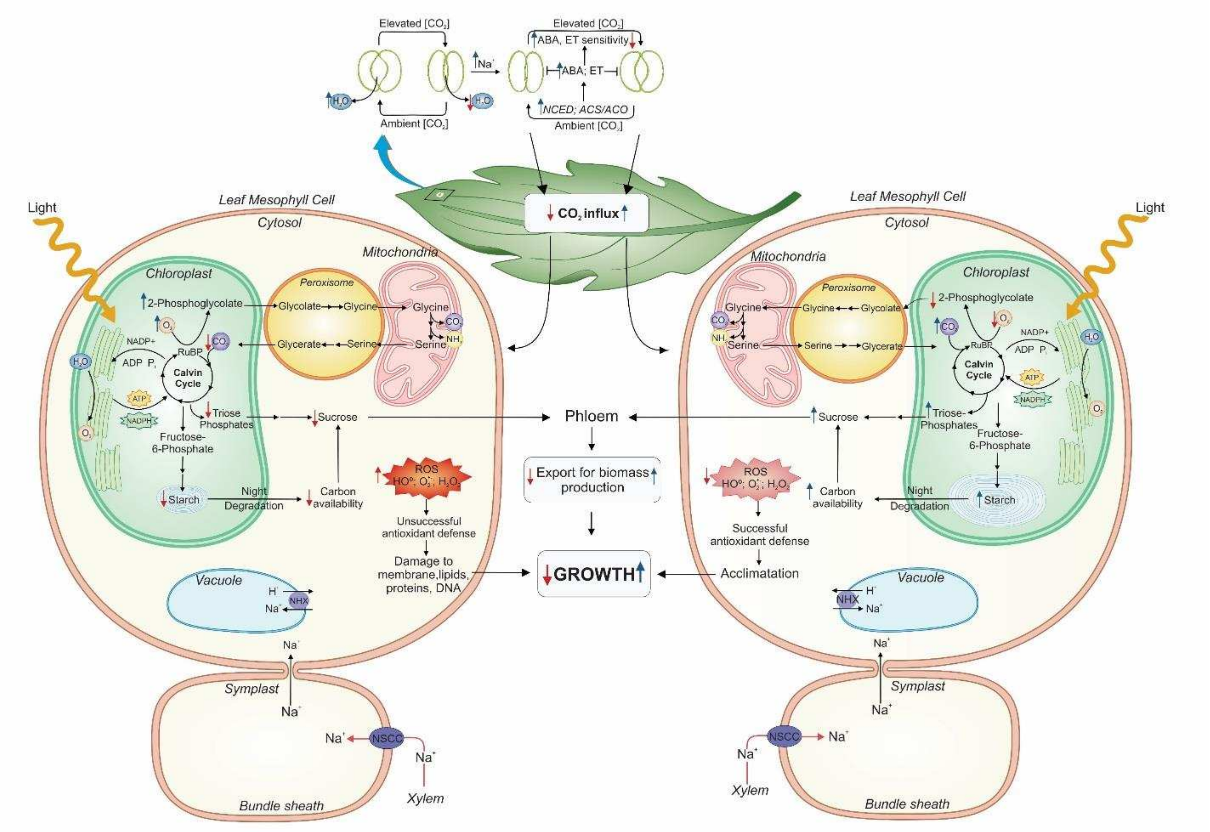

Figure 3. Salinity modifies the relationships between the availability of CO_2 and photoassimilate partitioning in leaves. Compared with ambient CO_2 concentration, high CO_2 increases carbon uptake, while often decreasing stomatal conductance in leaves of crops. Crop species respond to salinity by an increase in the expression of genes associated with ABA and ethylene biosynthesis. As a result, salt stress increases the production of ABA and ethylene, which induces stomatal closure an in turn, reduces assimilation rate of crops under ambient CO_2 concentration. However, leaves under elevated CO_2 concentration respond to NaCl exposure with marginal ABA and ethylene accumulation, adjusting the size of the stomatal pore. Consequently, the ABA and ethylene sensitivity of the guard cell should differ between crops under ambient and elevated CO_2 concentration. Under ambient CO_2 concentration, lower Calvin cycle activity in crops grown under salinity conditions results in less energy production and less $NADP^+$ regeneration, leading to over-reduction of the photosynthetic electron transport chain and ROS production. Thus, the oxygenase reaction of RubisCO and photorespiration represent a waste of energy and carbon. In addition, enhanced photorespiration of salt-stressed plants under ambient CO_2 concentration produces an additional load of ROS, thereby perturbing enzyme, cell wall and membrane functions. On the other hand, high CO_2 leads to higher rate of photosynthesis increasing the availability of carbohydrate in leaves of crops under salt stress. Hence, carbohydrates export is increased in elevated CO_2 concentration in mature leaves resulting increase in growth and crop yield. The mechanism of salinity action in response to increased atmospheric CO_2 concentration can differ, depending on the concentration of salt, the duration of exposure, the age of the plant, and the genotype. Abbreviations: ABA, abscisic acid; ACO, 1-aminocyclopropane-1-carboxylic acid oxidase, ACS, 1-aminocyclopropane-1-carboxylic acid synthase, ET, ethylene; NCED, nine-cis-epoxycarotenoid dioxygenase; NHX, Na^+/H^+ exchanger; NSCC, nonselective cation channels; ROS, reactive oxygen species; RuBP, ribulose-1,5-bisphosphate.

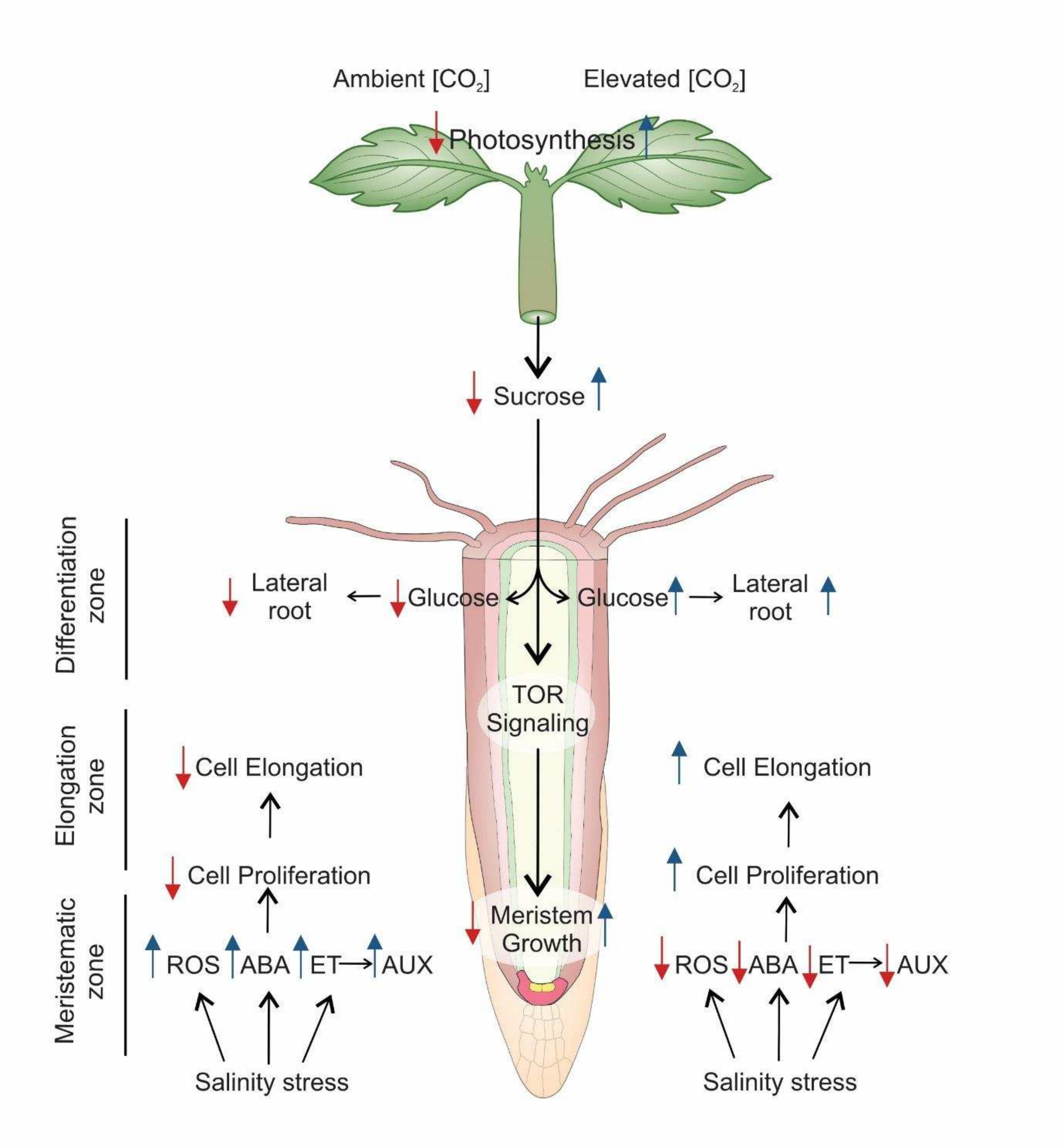

Figure 4. Salt stress and the control of root growth under ambient and elevated CO_2 concentration. Sugars exported from the shoot to root regulate root system development in response to salt stress. Source leaves maintain sucrose production and phloem export during salt stress to support the development of root system, even though rates of photosynthesis and leaf growth are reduced. Here, sugars content in leaves of crops grown under ambient CO_2 concentration is much lower than in crops under high CO_2, which suggests that the effects of sugars on root development are compromised in plant grown under ambient CO_2 concentration compared to elevated CO_2. Abbreviations: ABA, abscisic acid; AUX, auxin; ET, ethylene; ROS, reactive oxygen species, TOR, Target of Rapamycin.

CHAPTER 1

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Elevated CO_2 improves assimilation rate and growth of tomato plants under progressively higher soil salinity by decreasing abscisic acid and ethylene levels

Highlights

- Salt stress stimulates ABA and ACC biosynthesis in tomato plants
- The [eCO_2] determines the stomatal responses to ABA and ACC under salt stress
- The [eCO_2] decreases ABA and ACC biosynthesis in tomato plants under salt stress
- Metabolic alterations contributes to the restoration of plant growth under salinity in [eCO_2]

Abstract

High salinity in the soil hampers crop performance. In this context, elevated atmospheric CO_2 concentration can alter plant responses to excess salinity, as shown by studies exposing plants to high concentration of NaCl (osmotic shock) in controlled conditions under constant irradiance and temperature. These experiments, however, may not reflect the conditions that occur in natural environments. Here, we studied the impact of progressively imposed salt stress on tomato plant biomass, hormone biosynthesis and primary metabolism under elevated CO_2 concentration with fluctuating irradiance and temperature. Na^+ concentration and Na^+:K^+ ratios in plant tissues were increased by high salinity under both ambient and elevated CO_2 concentration. Elevated CO_2 led to enhanced growth in tomato plants under salt stress through stimulation of photosynthesis and reduced concentrations of abscisic acid and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid in both leaves and roots. Furthermore, whereas high salinity reduced the concentration of Krebs Cycle intermediates and increased the concentration of photorespiratory metabolites glycine and serine, all were restored to control levels by elevated CO_2. Our results demonstrate a beneficial effect of rising atmospheric CO_2 concentration for crops and reveal new interactions in the physiological network controlling salinity stress responses.

Keywords:

Climate change; hormones; primary metabolism; salt stress; Solanum lycopersicum

Abbreviations:

ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; aCO_2, ambient [CO_2]; eCO_2, elevated [CO_2]; A, net CO_2 assimilation rate, C_i/C_a, internal-to-ambient CO_2 concentration ratio; E, transpiration rate; g_S, stomatal conductance; NPQ, non-photochemical quenching coefficient; R_D, mitochondrial respiration rate; DW, dry weight; qRT-PCR, quantitative real-time PCR; TCA, tricarboxylic acid.

1 Introduction

Soil and water salinity represent a serious challenge for world agriculture. Salt hampers plant growth due to osmotic effects, ion toxicity, restriction of gas exchange capacity and nutrient imbalances in the cytosol (Ismail and Horie, 2017; Stepien and Johnson, 2009). On the other hand, elevated CO_2 concentration ([eCO_2]) often leads to stimulation of photosynthesis and growth potential of C3 plants (Kant et al., 2012; Morales et al., 2018). In this context, there is extensive evidence, obtained from experiments conducted in controlled environmental chambers, that the negative effects of salinity on plant growth may be mitigated by [eCO_2] (Piñero et al., 2016, 2014; Teng et al., 2006; Yi et al., 2015). However, plants growing in natural conditions are exposed to frequent diurnal fluctuations in irradiance and temperature. Plants in natural environments show clear differences on primary metabolism and growth compared to plants grown in controlled environments (Annunziata et al., 2017). Therefore, an important question is how central metabolism and plant growth under salt stress condition are adjusted with the availability of [eCO_2] in an environment with frequent changes in irradiance and temperature.

The ameliorative effect of [eCO_2] on tomato plants suffering from salinity stress is associated with reduced transpiration rates, which leads to decreased leaf Na^+ concentration (Yi et al., 2015). However, stomatal conductance (g_s) tends to increase as leaf temperature increases under both [aCO_2] and [eCO_2] (Urban et al., 2017). As a result, higher evaporative cooling can revert the negative impacts of supra-optimal ambient temperature on photosynthetic performance during heat waves in plants under well-watered conditions (Ameye et al., 2012). Notably, heat stress ameliorates the effect of salinity in tomato plants by maintaining the Na^+/K^+ homeostasis that, in turn, enhances photosynthetic rate per unit leaf area as compared with salinity alone (Rivero et al., 2014). On the other hand, high temperature decreases growth (sink activity) of tomato plants under salt stress by inhibiting leaf expansion and internode length (Chen et al., 2015; Najla et al., 2009). These experimental results suggest that growth itself (sink activity) is more limited than carbon availability (source activity). There is compelling evidence that higher CO_2 concentration leads to stimulation of plant growth by influencing cell division and cell expansion (Luomala et al., 2005; Taylor et al., 2003). Moreover, C3 photosynthesis is usually not saturated at current [aCO_2] (Busch and Sage, 2017; Sharwood et al., 2016). Thus, it seems likely that [eCO_2] would rebalance growth and carbon supply of tomato plants grown under high soil salinity.

Hormones play an important role in controlling plant-environment interactions (Nguyen et al., 2016). For example, salt stress leads to stimulation of ethylene and abscisic acid (ABA) biosynthesis, which can directly inhibit growth by negatively regulating cell expansion (Silva et al., 2018; Park et al., 2012). The accumulation of ABA under high salt also induces stomatal closure, decreasing the rate of leaf gas exchange and growth of tomato plants (Lovelli et al., 2012). Interestingly, it was shown that [eCO_2] could inhibit ABA production in roots of sweet pepper plants grown under salt stress, which, in turn, is correlated with the stomatal response (Piñero et al., 2014). Ethylene and its direct precursor 1-aminocyclopropane-1-carboxylic acid (ACC) also influence stomatal closure (Desikan et al., 2006; He et al., 2011). Salinity stimulates ACC biosynthesis, which impairs growth of tomato plants (Albacete et al., 2009). Moreover, ACC accumulation in tomato leaves appears to be associated with the onset of oxidative stress and the decline in chlorophyll fluorescence under salt stress (Ghanem et al., 2008). Intriguingly, [eCO_2] may influence the ethylene signaling pathway in a yet unknown way (Guo et al., 2014). Overall, these observations indicate that the regulation of ABA and ethylene biosynthesis by [eCO_2] provides a framework to understand how plant growth is adjusted under salt stress.

Here, we investigated the response of tomato plants to a combination of progressively higher soil salinity and [eCO_2] in an environment with fluctuating irradiance and temperature. We focused on the hormonal (ABA and ethylene), metabolic (amino acids and Krebs Cycle intermediates) and physiological (photosynthesis, respiration) changes induced by gradual exposure to increasing concentration of NaCl during growth of tomato plants under [eCO_2]. We discuss the potential impact of rising [aCO_2] on the plants' response to high salinity and how this knowledge could be exploited to engineer salt tolerance in crops.

2 Materials and methods

2.1 Plant material and experimental design

Seeds of tomato plants (Solanum lycopersicum L.) cv. Santa Clara were sown on a 1:1 (v/v) mixture of commercial substrate (Tropstrato HT, Mogi Mirim, Brazil) and vermiculite supplemented with 1 g l^{-1} 10:10:10 NPK and 4 g l^{-1} dolomite limestone ($MgCO_3$ + $CaCO_3$) in plastic pots with a 3.5 l capacity. Plants were first established by growth under [aCO_2] in a greenhouse at the Universidade Federal de Viçosa (20^{o}45'S, 42^{o}15'W, 650 m altitude) in southeastern Brazil. When the first true leaves appeared, seedlings grown singly in pots were watered with deionized water (control) or 50 mM NaCl solution. Afterwards, NaCl concentration was gradually increased by 50 mM every 2 days, until NaCl concentrations reached 150 mM. Two days after reaching this concentration, plants used in the assays were placed in open-top chamber (4.2 m diameter and 2.4 m high) in a random arrangement with 18 pots per chamber under either [aCO_2] (400 ± 20 µmol mol^{-1}) or [eCO_2] (750 ± 30 µmol mol^{-1}). Plants in the open-top chambers were watered at 2-days intervals with deionized water (control) or 150 mM NaCl solution for 21 days. Three independent experiments were carried out from June 2016 to August 2018 in open-top chambers inside the same greenhouse of Universidade Federal de Viçosa, under natural photoperiod. The temperature, relative humidity and photosynthetic photon flux density (PPFD) inside the chambers were recorded at intervals of 30 min by sensors coupled to a data logger (Li-1400, LI-COR, Lincoln, NE, USA). Environmental conditions (DLI, daily light integral; VPD, vapour pressure deficit; and air temperature) inside the chambers during the course of the experiment are shown in Fig. S3. The CO_2 level in the enriched chamber was maintained by constantly injecting of CO_2 (99.8 %) from a compressed gas cylinder into the chamber. Moreover, the CO_2 levels were monitored using an infrared gas analyser (Li-840, LI-COR, Lincoln, NE, USA).

2.2 Vegetative growth assessment

The number of leaves on the main stem with a length > 1 cm was recorded. At each harvest, the roots, stem and leaves of each plant were separated with scissors and the leaf area was determined by using a Li-Cor 3100 area meter (LI-COR, Lincoln, NE, USA). Root systems were collected using the procedure as described by Ribeiro et al. (2016). Afterwards, the roots, stems and leaves of each plant were oven dried for at least 3 days at 70 ^oC for determining dry weight (DW). The relative growth rate (RGR) was calculated from the equation (Hunt, 1990): $RGR = [(\ln W_2 - \ln W_1)/(t_2 - t_1)]$, where W_1 and W_2 are plant dry weights at time t_1 and t_2. Tomato plants were harvested at day 1 (t_1) and 21 (t_2) after transfer to the open-top chambers.

2.3 Gas exchange and chlorophyll fluorescence measurements

The net CO_2 assimilation rate (A), intercellular-to-atmospheric CO_2 ratio (C_i/C_a), stomatal conductance (g_s), transpiration rate (E), mitochondrial respiration rate in the dark (R_D) were determined simultaneously with chlorophyll a fluorescence measurements using an open-flow gas exchange system infrared gas analyzer (LI-6400XT, LI-COR, Lincoln, NE, USA) equipped with an integrated with a fluorescence chamber head (LI-6400-40, LI-COR, Lincoln, NE, USA). The A and g_s measurements were made on the third fully expanded leaf between 09:00 and 11:30 h. The values of the intercepted PPFD, measured with a photometer/radiometer (Li- 185, LI-COR, Lincoln, NE, USA) were, on average, 1000±102 μmol m^{-2} s^{-1} at the leaf level. Therefore, measurements of gas exchanges were conducted using the average values of PPFD (mentioned above) intercepted by the sampled leaves in their natural angles. Moreover, the conditions in the leaf chamber consisted of a VPD of 1.0 kPa and air temperature 25 ^oC, which corresponded to the average values of VPD and temperature obtained inside the open-top chambers. The CO_2 concentration in the cuvette was set at 400 μmol CO_2 mol^{-1} air (for plants under [aCO_2]) and 750 μmol CO_2 mol^{-1} air (for plants under [eCO_2]) using a CO_2 injector and compressed CO_2 cartridge. The R_D was measured using the same gas exchange system described above after at least two hours into the dark period in the same leaf previously used to determine gas exchange parameters.

The background fluorescence signal (F_0) was determined illuminating dark-adapted leaves (2 h) with weak modulated measuring beams (0.03 µmol m^{-2} s^{-1}). The maximum fluorescence (F_m) was measured with a saturating white light pulse of 8000 µmol m^{-2} s^{-1} for 0.8 s. The maximal efficiency of PSII photochemistry was calculated: $F_v/F_m=[(F_m - F_0)/F_m)]$. The coefficient of non-photochemical quenching (NPQ) was calculated as previously described (Martins et al., 2014). The A/C_i curves were determined as previously detailed (Long and Bernacchi, 2003; Pérez-López et al., 2012).

2.4 Metabolite analysis

Roots and fully expanded third leaves of tomato plants were harvested at the end of the photoperiod and immediately frozen in liquid nitrogen and stored at –80 ^oC until analysis. Sucrose, fructose and glucose were measured in ethanol-water extracts prepared from freeze-dried and homogenized sample material of roots and leaves, approximately 50 mg dry weight, as described by Cross et al. (2006). Starch and total protein levels were determined for the residual fractions of ethanol-water extracts suspended in NaOH 100 mM. Protein levels were assessed with the Bio-Rad Bradford reagent (Bio-Rad Laboratories) according to the manufacturer's instructions. Assays were performed in 96-well microplates and absorbance was determined using a VersaMax ELISA microplate reader (Molecular Devices, Sunny Valle, EUA).

Extraction and derivatization of metabolites from tissues for gas chromatography-mass spectrometry (GC-MS) were performed exactly as described by Lisec et al. (2006). The GC-MS system was comprised of an AOC-20i autosampler and a QP2010 SE gas chromatograph-quadrupole mass spectrometer (Shimadzu, Tokyo, Japan) equipped with a column Rtx-5MS (Restek, Bellefonte, CA, USA). The GC-MS analysis were performed as described previously in Luedemann et al. (2008).

2.5 Na^+ and K^+ concentrations

Soluble ions (Na^+ and K^+) were extracted and assayed as described by Munns et al (2010). The dried plant samples (roots, stems and leaves) were ground using a Wiley mill (Cienlab CE-438, 8 blades, 1,725 rpm 20 mesh size) and approximately 0.5 g of each samples were digested with $HNO_3/HClO_4$ (2:1, v/v). Na^+ and K^+ concentrations were determined by using an inductively coupled plasma optical emission spectrometer (ICP-OES, Perkin Elmer Optima 3000 XL).

2.6 ABA and ACC analysis

ABA and ACC was quantified using the methods described by Müller and Munné-Bosch (2011). The samples were automatically injected (5 µL) in the system LC - MS/MS using an Agilent 1200 Infinity Series coupled to a Mass Spectrometry type triple Quadrupole (QqQ), model 6430 (Agilent Technologies, Palo Alto, CA, USA). Chromatographic separation was carried out on a column Zorbax Eclipe Plus C18 (1.8 µm, 2.1 x 50 mm; Agilent Technologies, Palo Alto, CA, USA) in series with a guard column Zorbax (SB C18, 1.8 µm; Agilent Technologies, Palo Alto, CA, USA).

2.7 Gene expression quantification

Total RNA was isolated from leaves of tomato plants using an RNeasy Plant Mini Kit (Qiagen) and cDNA was synthesized from 2 µg total RNAs using SuperScript TM III reverse transcriptase (Invitrogen), following the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) reactions were performed as described by Zanor et al. (2009). Relative transcript abundance was calculated by the comparative cycle threshold (CT) method (Livak and Schmittgen, 2001). Each sample was normalized using ACTIN as an internal control. The gene-specific primers used for qRT-PCR are shown in Table S1.

2.8 Stomatal aperture bioassays

Lateral leaflets of the 3rd leaf from tomato plants were floated in buffer solution (10 mM MES, 5 mM KCl, 50 µM $CaCl_2$, pH 6.15) with abaxial side down in Petri dishes under light for 2.5 h. Following this, leaflets were treated with 100 µM ABA, 100 µM ACC and 100 µM ABA+100 µM ACC supplemented in the same buffer medium for a further 2.5 h. After ABA and/or ACC treatment, the stomatal apertures were performed as described by Ribeiro et al. (2009).

2.9 Statistical analysis

The experiments were designed in a completely randomized distribution. For quantification of metabolites, ACC and ABA six independent samples containing five leaves or five root systems per samples were assayed. For real-time qPCR analysis three independent replicates were assayed. Two-way analysis of variance (ANOVA, $P < 0.05$) was used to determine effects of salt and CO_2 concentration. Difference among treatment means ($P < 0.05$) was evaluated using Tukey or t-test. Scott-Knott test ($P < 0.05$) was used for comparison data of stomatal aperture width. All statistical analyses were performed using the SPSS statistical package.

3 Results

3.1 Enhanced growth of salt-treated plants under [eCO_2] is not associated with inhibition of Na^+ transport from roots to leaves

Salt stress decreased growth of tomato plants under [aCO_2], which was restored by high CO_2 concentrations (Fig. 1A). Elevation of CO_2 concentration led to an increase of dry weight of leaves, stems and roots as well as total biomass and total leaf area in tomato plants grown in soil without NaCl supplementation (-NaCl) (Fig. 1B-F). However, the RGR was not significantly affected in -NaCl plants under [eCO_2] as compared to plants under [aCO_2] (Fig. 1G). Moreover, the inhibitory effect of salt stress on growth of tomato plant under [aCO_2] was overcome in plants grown at elevated [CO_2] (Fig. 1A-G).

In order to investigate whether [eCO_2] could promote a selective control on the ion absorption, we measured the levels of Na^+ and K^+ in leaves, stems and roots. Plants treated with NaCl showed a marked increase in tissue Na^+ levels under both CO_2 concentrations (Fig. 2A-C). Moreover, treatment of plants with either [aCO_2] or [eCO_2] did not alter the levels of Na^+ in leaves, stems and roots under non-saline or saline conditions. The levels of K^+ in leaves did not differ between -NaCl and +NaCl plants under both CO_2 concentrations (Fig 2D). Additionally, the levels of K^+ in stems and roots were unaffected in all treatments (Fig. 2E-F). NaCl treatment led to increased Na^+:K^+ ratio, but the CO_2 concentrations did not influence the Na^+:K^+ ratio either in non-saline or saline conditions (Fig. 2G-I).

3.2 Salt stress modifies the biosynthesis of ABA and ACC in response to [eCO_2]

Salt stress increased concentrations of ABA and ACC, the immediate precursor of ethylene, in leaves and roots of tomato plants grown under both CO_2 concentrations (Fig. 3A, B). Moreover, NaCl promoted a larger increase of ABA and ACC in leaves and roots of tomato plants grown at [aCO_2] than at [eCO_2]. The expression of ethylene biosynthesis genes ACS1, ACS2, ACO1 and ACO4 as well as ABA biosynthesis genes NCED1 and ZEP was upregulated in leaves of tomato plants treated with NaCl both under [aCO_2] and [eCO_2] (Fig. 3C). However, the expression of genes associated with biosynthesis of ethylene and ABA was lower in plants treated with NaCl grown under [eCO_2] as compared with plants at [aCO_2].

3.3 Changes on photosynthetic performance and stomatal aperture are interlinked with saline condition and [eCO_2]

Under [aCO_2] several gas-exchange parameters (i.e., A, C_i/C_a, g_s, E and R_D) were decreased by the NaCl treatment but no difference was observed for these parameters under [eCO_2] (Fig. 4A-E). Moreover, NPQ decreased in plants treated with NaCl under [eCO_2]

compared with plants under [aCO_2], while NPQ was not significantly affected in –NaCl plants under both CO_2 concentrations (Fig. 4F). On the other hand, the maximum rate of carboxylation (V_{cmax}), maximum rate of carboxylation limited by electron transport (J_{max}) and F_v/F_m were neither significantly affected by the NaCl treatment under both CO_2 concentrations (Fig. S1). In our experimental setup, the specific leaf area (SLA) stomatal index and chlorophyll levels were not affected by the treatments performed (Fig. S1).

In general, ABA and/or ACC treatment resulted in a decrease on the stomatal aperture in leaves of tomato plants grown under control and saline conditions under both [aCO_2] and [eCO_2] (Fig. 4G). However, the stomatal apertures of leaves upon salt stress treated with either ABA or ACC were found to be 32% and 36% greater, respectively, under [eCO_2] than in [aCO_2]. Under salt stress conditions, ABA treatment combined with ACC treatment also further reduced the stomatal aperture of plants under [aCO_2] compared with plants under [eCO_2] (Fig. 4G).

3.4 Changes in metabolite profiles in leaves and roots of tomato plants in response to salt stress and [eCO_2]

In non-saline condition, [eCO_2] did not significantly influence the levels of hexoses in both leaves and roots, as compared with tomato plants grown under [aCO_2] (Fig. 5A). Moreover, hexoses levels decreased by 36% in leaves of plants treated with NaCl under [eCO_2] compared with plants under [aCO_2]. In contrast to the observed in leaves, the levels of hexoses in roots of plants treated with NaCl under [eCO_2] were higher than plants under salt stress condition at [aCO_2] (Fig. 5A). Treatment with NaCl solution tended to increase sucrose levels in leaves and roots under both CO_2 concentrations compared with control (–NaCl) under [aCO_2] (Fig. 5B). Under [eCO_2], starch levels increased in leaves and roots of plants grown under both non-saline and saline conditions compared with untreated plants under [aCO_2] (Fig. 5C). Salt stress did not influence the levels of protein in roots under both CO_2 concentrations compared with untreated plants (–NaCl) (Fig. 5D). There was only a small decrease in protein levels for leaves of plants treated with NaCl under [eCO_2] compared with control (–NaCl) under [aCO_2] (Fig. 5D).

Under salt stress conditions, there was an increase in levels of alanine, asparagine, aspartate, glycine, glutamate, glutamine, serine and tyrosine in leaves of plants under [aCO_2], whereas those amino acids showed a decrease under [eCO_2] compared with control (–NaCl) under [aCO_2] (Fig. 6A). There was an increase of arginine in leaves of plants treated with NaCl under [aCO_2], but not under [eCO_2]. Levels of valine, tryptophan, threonine, lysine, methionine, ornithine, phenylalanine, GABA and cysteine in leaves were unaltered between treatments under both CO_2 concentrations (Fig. 6A). Salt stress increased levels of proline in leaves of plants grown under both CO_2 concentrations compared with control (–NaCl) under [aCO_2]. On the other hand, levels of alanine, arginine, asparagine, aspartate, GABA, glycine, glutamate, glutamine, serine and threonine increased significantly in roots of plants treated with NaCl under [aCO_2], while these amino acids remained at the same levels in roots under [eCO_2] concentrations compared with control (–NaCl) under [aCO_2] (Fig. 6B). Moreover, salt stress decreased the tryptophan levels in roots of plants grown under [aCO_2], while tryptophan was unaltered by NaCl in roots under [eCO_2]. Proline levels were increased in roots of plants treated with NaCl under both CO_2 concentrations compared with control (–NaCl) under [aCO_2]. Levels of lysine, methionine, ornithine and phenylalanine in roots remained steady across all treatments (Fig. 6B).

Elevation of CO_2 concentration had no effect in the levels of TCA cycle intermediates, namely, oxaloacetate, isocitrate, 2-oxoglutarate, fumarate and malate in leaves of tomato plants untreated with NaCl, whereas the levels of citrate and succinate were increased in such plants compared with control (–NaCl) under [aCO_2]. (Fig 7A). A significant increase in citrate level was also observed in roots of tomato plants untreated with NaCl under [eCO_2] (Fig. 7B). Moreover, the levels of isocitrate, 2-oxoglutarate, succinate, fumarate, malate and oxaloacetate did not differ in roots of plants untreated with salt under [eCO_2] compared with control (–NaCl) under [aCO_2] (Fig. 7B). Interestingly, salt stress resulted in decreased levels of isocitrate, 2-oxoglutarate, fumarate and malate in leaves of plants grown under [aCO_2], however, [eCO_2] restored these metabolites to control levels (Fig. 7A). The levels of oxaloacetate, citrate and succinate in leaves were reduced by salt stress under [aCO_2], but increased during [eCO_2] conditions compared with untreated plants (–NaCl) under [aCO_2] (Fig. 7A). In roots, the levels of citrate, isocitrate and 2-oxoglutarate were increased by NaCl treatment under [aCO_2] (Fig. 7B). On the other hand, salt stress decreased the levels of succinate, fumarate, malate and oxaloacetate in roots of plants grown under [aCO_2] compared with untreated plants (–NaCl) under [aCO_2]. The levels of isocitrate, 2-oxoglutarate, succinate, fumarate, malate and oxaloacetate in roots of plants treated with NaCl under [eCO_2] were similar to those observed in plants untreated with salt under [aCO_2] (Fig. 7B).

4 Discussion

Plant responses to [eCO_2] and salinity have been mostly investigated in controlled environment chambers under constant irradiance and temperature (Pérez-López et al., 2015; Piñero et al., 2014; Zaghdoud et al., 2013). However, plants grown in controlled environments can show different responses from plants grown in natural conditions (Annunziata et al., 2017; Mishra et al., 2012). In our experimental setup with free fluctuation of irradiance and temperature, we found that [eCO_2] leads to a stimulation of photosynthesis, metabolic homeostasis and increases growth in tomato plants under salt stress. These changes were accompanied by reductions in concentrations of ABA and ACC, the precursor of ethylene, and their respective biosynthesis genes.

There is evidence that salinity increases the level of ABA in tomato leaves, which causes stomatal closure (Lovelli et al., 2012). Moreover, ACC also induces stomatal closure in Arabidopsis (Desikan et al., 2006) and tomato (Borbély et al., 2019). Under salt stress conditions, leaves of plants grown under [eCO_2] retained 52 % and 62 % of the concentration of ABA and ACC, respectively, of leaves under [aCO_2] (Fig. 3). However, gs increased in plants treated with NaCl under [eCO_2], when compared with plants incubated with salt under [aCO_2] (Fig. 4). This might indicate a certain guard cell flexibility of plants grown under [eCO_2], which allows for an increased stomatal aperture under salt stress conditions. In fact, treatment of leaflets of the 3rd leaf from tomato plants grown under [eCO_2] with ABA and/or ACC has less effect on stomatal closure under salt stress conditions (Fig. 4).

It was previously reported that the decline of g_s under [eCO_2] improves salt tolerance in tomato plants by decreasing root-to-shoot Na^+ transport and, thus, the accumulation of Na^+ at toxic levels in leaves (Yi et al., 2015). Our data from tomato plants grown under fluctuation of irradiance and temperature indicate that [eCO_2] kept the g_s and E of plants treated with NaCl similar to the values observed in plants grown without salt stress under [aCO_2] (Fig. 4). As a result, NaCl treatment did not alter Na^+ levels and Na^+:K^+ ratio in plants growth under both [aCO_2] and [eCO_2] (Fig. 2). It has been found that elevation of leaf temperature increased g_s under both [aCO_2] and [eCO_2] (Urban et al., 2017). However, our analyses revealed that salt stress did not change leaf temperature under both CO_2 concentrations (Fig. S2). Raising atmospheric vapour pressure deficit (VPD) causes an increase in ABA biosynthesis in Angiosperms (McAdam and Brodribb, 2016). We did not observe any drastic differences in VPD among chambers (Fig. S3). Hence, the increase in gs and E was not caused by differences in leaf temperature and VPD, but was driven by decreases in concentrations of ABA and ACC in leaves of plants treated with NaCl under [eCO_2] associated to the limited effect of ABA and ACC on stomatal closure under [eCO_2].

High soil salinity has a major influence on the plants photosynthetic performance (Wang et al., 2018). In our study, salt stress did not lead to significant changes in the NPQ in plants grown under either [aCO_2] or [eCO_2] compared with control plants (–NaCl) under [aCO_2] (Fig. 4). In addition, the high J_{max} values measured even for plants in which A was impaired indicates that the capacity for synthesis of both ATP and NADPH required to fuel carbon fixation reactions was not affected in plants grown under saline conditions. Furthermore, V_{cmax} was not altered by salt stress in plants grown under both CO_2 concentrations (Fig S1), showing the maintenance of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) capacity to fix CO_2. Thus, restoration of plant growth under salt stress by [eCO_2] may be related to a decreased photosynthetic limitation by CO_2 diffusion mediated by lower concentrations of ABA and ACC. Consistent with this view, ABA-deficient tomato mutant showed increased g_s and C_i (Bradford, 1983). Moreover, the low g_s of tomato plants treated with 100 µM ACC resulted in a low C_i (Borbély et al., 2019). Under [aCO_2], dark respiration decreased more than photosynthesis under salt stress (Fig 4). There was a stimulation of A in plants treated with NaCl under [eCO_2], while dark respiration remained similar to control (-NaCl) under [aCO_2] (Fig. 4). These results suggest that salinity was not primarily associated with down-regulation of carbon metabolism, independently of CO_2 concentration. In fact, starch accumulation at the end of the day was similar in controls (-NaCl) and plants treated with NaCl under [aCO_2] or [eCO_2] (Fig. 5). In this context, our results differ from part of the literature, which suggests carbon shortage under salt stress (Pérez-López et al., 2012; Reef et al., 2015), most likely in response to a rapid imposition of salt stress. However, in our study salt stress was progressively established by increasing NaCl concentrations under stress conditions, which could have contributed to metabolic adaptation to the full salt concentration. Indeed, the metabolic adjustment was reported to be apparent when salt stress was imposed progressively in Zea mays (Richter et al., 2015).

Sugars, amino acids and organic acids have a central regulatory function in the control of plant growth (Smith and Stitt, 2007). Biomass was increased in NaCl-treated plants growth under [eCO_2] as compared to [aCO_2] (Fig. 1). This was due to a significant increase in the RGR (Fig 1), suggesting an improvement of the fluxes in central carbon and nitrogen metabolism under salt stress. Our analysis indicates that the levels of sucrose in leaves and roots tends to increase under salt stress both under [aCO_2] and [eCO_2] (Fig. 5). However, the high levels of amino acids in leaves and roots of plants treated with NaCl grown under [aCO_2] were not maintained under [eCO_2] (Fig. 6), indicating that amino acids are likely being a source of carbon and nitrogen for growth under [eCO_2]. Arginine and asparagine act as an important nitrogen storage compound in plants (Winter et al., 2015). The increased levels of arginine and asparagine in leaves and roots of plants treated with NaCl under [aCO_2] indicate that both amino acids are being utilized more slowly for growth of tomato plants (Fig. 6). This is supported by the decrease of the arginine and asparagine pool and increase in growth of plants treated with NaCl under [eCO_2]. There was also an increase in levels of aspartate, glutamine and glutamate in leaves and roots of plants treated with NaCl under [aCO_2], which fell under elevated CO_2 concentration (Fig. 6). Together, these data indicate that [eCO_2] permit an appropriated modulation of energy metabolism and growth under salt stress conditions. In this respect, alanine levels increased in leaves and roots of plants treated with NaCl grown under [aCO_2], which might contribute to explain the decrease in growth of tomato plant grown during salt stress under [aCO_2] since increased alanine levels has been associated with decreased growth of plants (Miyashita et al., 2007). In line with this, [eCO_2] increased growth and decreased the levels of alanine in tomato plants treated with NaCl (Figs 1 and 6). Proline is often associated with protective functions, despite the fact that proline can inhibit plant growth (Maggio et al., 2002). Ethylene and ABA leads to an increase of levels of proline in plants (Cui et al., 2015; Pál et al., 2018). In light of this, the shifted relationship observed between proline and growth in the NaCl-treated plants under [eCO_2] might be a result of reduction in ACC and ABA biosynthesis (Figs 1, 3 and 6).

Photorespiration can lead to the additional amounts of glycine and serine in leaves of C3 plants (Bräutigam and Gowik, 2016). The levels of glycine and serine increased in leaves of NaCl-treated plants [aCO_2] and decreased under [eCO_2] (Fig. 6). However, photorespiration was not significantly altered in plants treated with NaCl under [aCO_2] compared with control (Fig. S1). The activity of antioxidant enzymes (ascorbate peroxidase, glutathione reductase, catalase and superoxide dismutase) was unaffected in leaves of plants treated with NaCl under [aCO_2] or [eCO_2] (Fig. S2), indicating that salt stress was not primary associated with oxidative stress. The avoidance of oxidative stress may be further corroborated by the similar levels of protein and total chlorophyll in tomato leaves regardless of NaCl treatment (Figs 5 and S1). Our results differ from previous studies which suggest a major reprograming of antioxidant system under salt stress under both [aCO_2] and [eCO_2] (Pérez-López et al., 2012; Woodrow et al., 2017; Yi et al., 2015). However, several of those studies were performed by rapidly imposing salt stress and in controlled growth conditions.

The present data also show higher levels of glycine and serine in roots of NaCl-treated plants under [aCO_2] than in [eCO_2] (Fig 6). This was accompanied by an increase in level of GABA in roots of plants treated with NaCl under [aCO_2]. It is known that NaCl induces an increase in levels of GABA in roots of various plant species, including Arabidopsis (Renault et al., 2010) and tomato (Bolarín et al., 1995). Salt stress also led to increases biosynthesis of glycine and serine in plant roots (Wu et al., 2013). Thus, our results imply that there is a lesser effect of the salt stress on synthesis of glycine, serine and GABA as well as growth of tomato plants under [eCO_2]. Consistent with this view, [eCO_2] decreased the levels of glycine, serine and GABA and increased root growth of plants under salt stress as compared with plants under [aCO_2] (Figs 1 and 6).

The TCA cycle plays an important role in plant salt tolerance (Batista-Silva et al., 2018; Li et al., 2017). In roots, the alterations in the TCA cycle intermediates, namely, isocitrate, 2-oxoglutarate, succinate, fumarate, malate and oxaloacetate by NaCl treatment under [aCO_2] were not observed upon [eCO_2] (Fig. 7). Furthermore, NaCl treatment resulted in lower levels of TCA cycle intermediates in leaves of plants grown under [aCO_2], which were restored by [eCO_2] (Fig. 7). Interestingly, salinity led to an increase in levels of citrate, succinate and oxaloacetate in leaves of plants grown under [eCO_2], but did not affect dark respiration compared with control (Figs 4 and 7). Thus, it is possible that the large variety of metabolic pathways makes plant respiration flexible, particularly in a continuously changing environment. Since organic acids of the TCA cycle were increased in leaves of NaCl-treated plant under [eCO_2] as compared with plants under [aCO_2], more energy in the form of NADH, $FADH_2$ and ATP may be available to support plant growth under a saline condition. In agreement with these observations, the decrease in RGR, leaf area, dry weight in leaves, stems and roots of NaCl-treated plants grown under [aCO_2] was not found when NaCl-treated plants were grown in [eCO_2] (Fig. 1). It should be noted that under [eCO_2], plants treated with NaCl displayed a greater stimulation in growth of leaves, stems and roots as compared with plants under [aCO_2] (Fig. 1). The ability to increase sink size could partially explain the effect of [eCO_2] on plant growth in the saline environment. ACC and ABA can directly inhibit plant growth (Luo et al., 2014; Dias et al., 2010). Thus, the decrease in levels of ABA and ACC under [eCO_2] could contribute to the increase sink activity of tomato plants under salt stress conditions.

5 Conclusions

We have shown that [eCO_2] mitigates the effect of high soil salinity in tomato plants under variable irradiance and temperature conditions (Fig. 8). We ascribe this effect to a combination of reduced ABA and ACC, which leads to higher assimilation rate driven mostly by higher stomatal conductance (Fig. 8). Furthermore, metabolic alterations such as increased

Krebs Cycle intermediates and reduced glycine and serine may contribute to the restoration of plant growth under high salinity in [eCO_2] conditions. Thus, our study highlights the role of [eCO_2] in modulating plant growth under salt stress conditions through alterations in ABA and ACC, leading to metabolic homeostasis in leaves and roots of tomato plants (Fig. 8). Atmospheric CO_2 concentration has recently reached a record high level of 415 ppm and is expected to reach 685 ppm by 2050. Thus, crop breeding for salinity tolerance needs to be informed by this key environmental variable. Our results provide an insight into the physiological networks involved.

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Figures

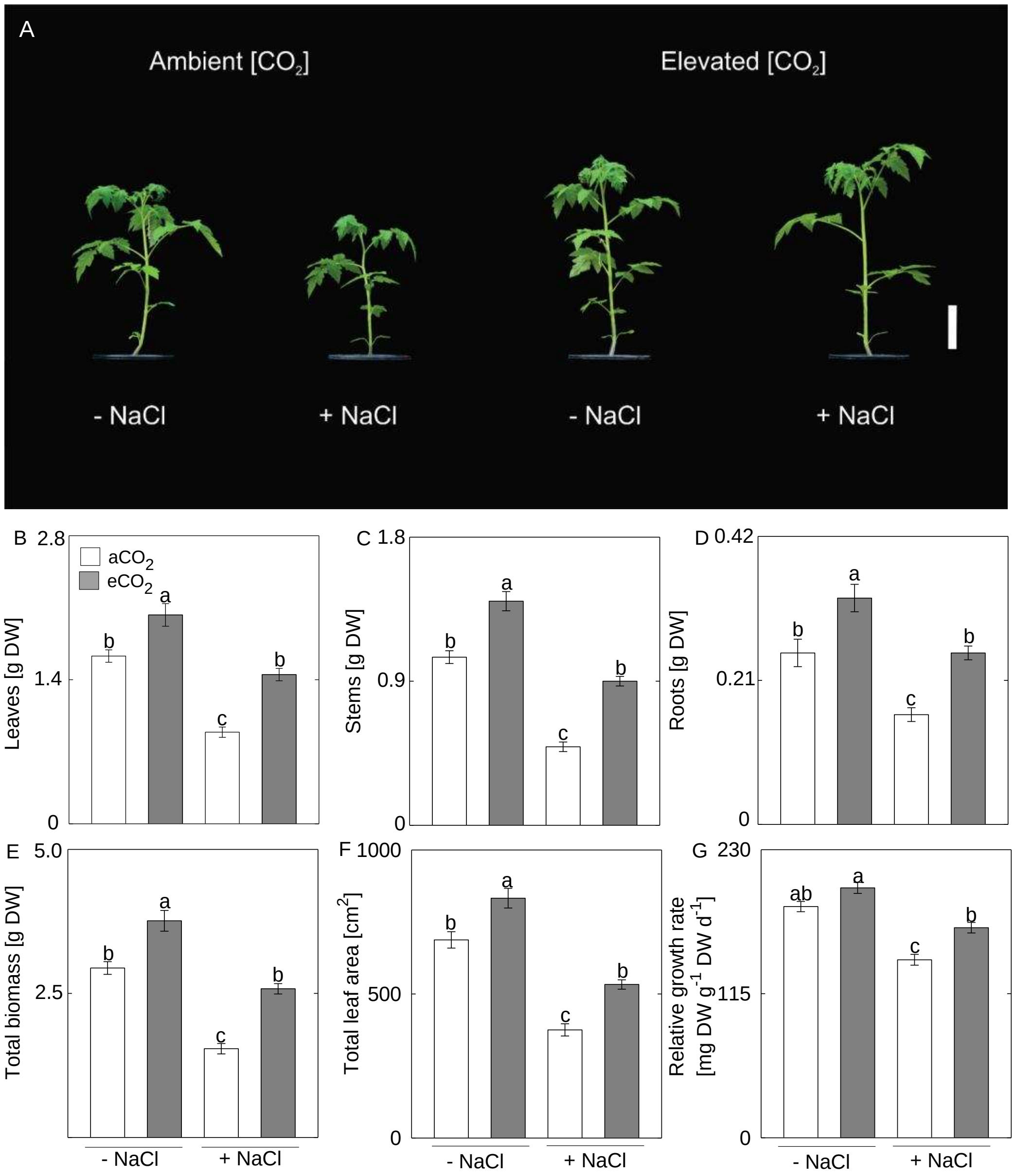

Fig. 1. Phenotypic changes of tomato plants caused by treatment with NaCl grown [aCO$_2$] and [eCO$_2$]. (A) Phenotype of tomato plants grown for 21 days under [aCO$_2$] and [eCO$_2$]. The scale bar represents 10 cm (B) Leaves dry weight. (C) Stems dry weight. (D) Roots dry weight. (E) Total plant dry weight. (F) Total leaf area. (G) Relative growth rate (on a dry weight basis). Bars labelled with the different letters indicate significant differences by Tukey's test at 5% probability. Data are the means ± standard error of three separate experiments, and in each experiment 10 plants were harvest for each treatment shown.

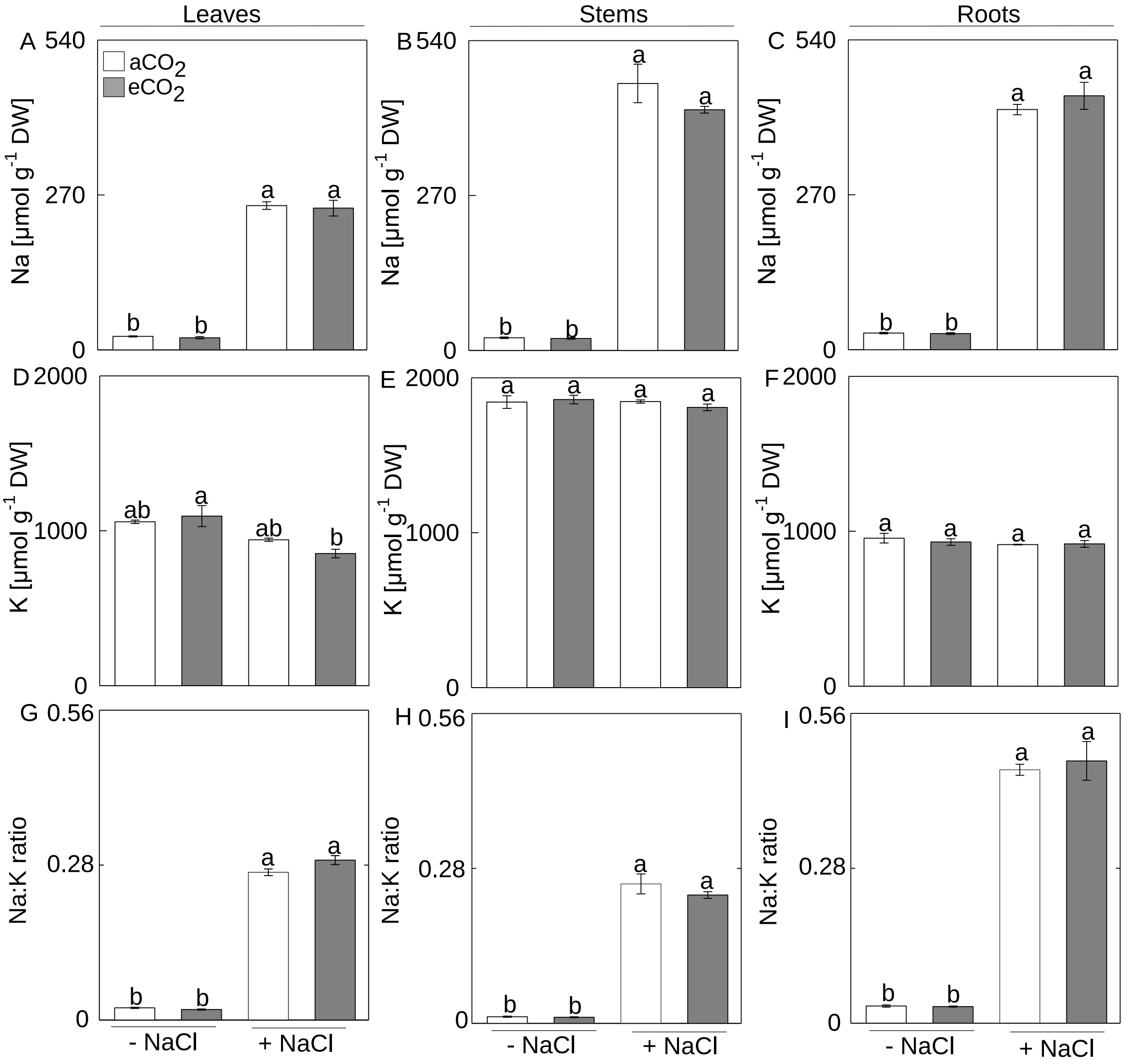

Fig. 2. Concentrations of Na^+, K^+ and Na^+:K^+ ratio in leaves, stems and roots of tomato plants treated with NaCl grown under [aCO_2] and [eCO_2]. (A-C) Na^+ in leaves, stems and roots. (D-F) K^+ in leaves, stems and roots. (G-I) Na:K ration in leaves, stems and roots. Bars labelled with the different letters indicate significant differences by Tukey's test at 5% probability. Data are means ± standard error of three separate experiments, with six replicates each.

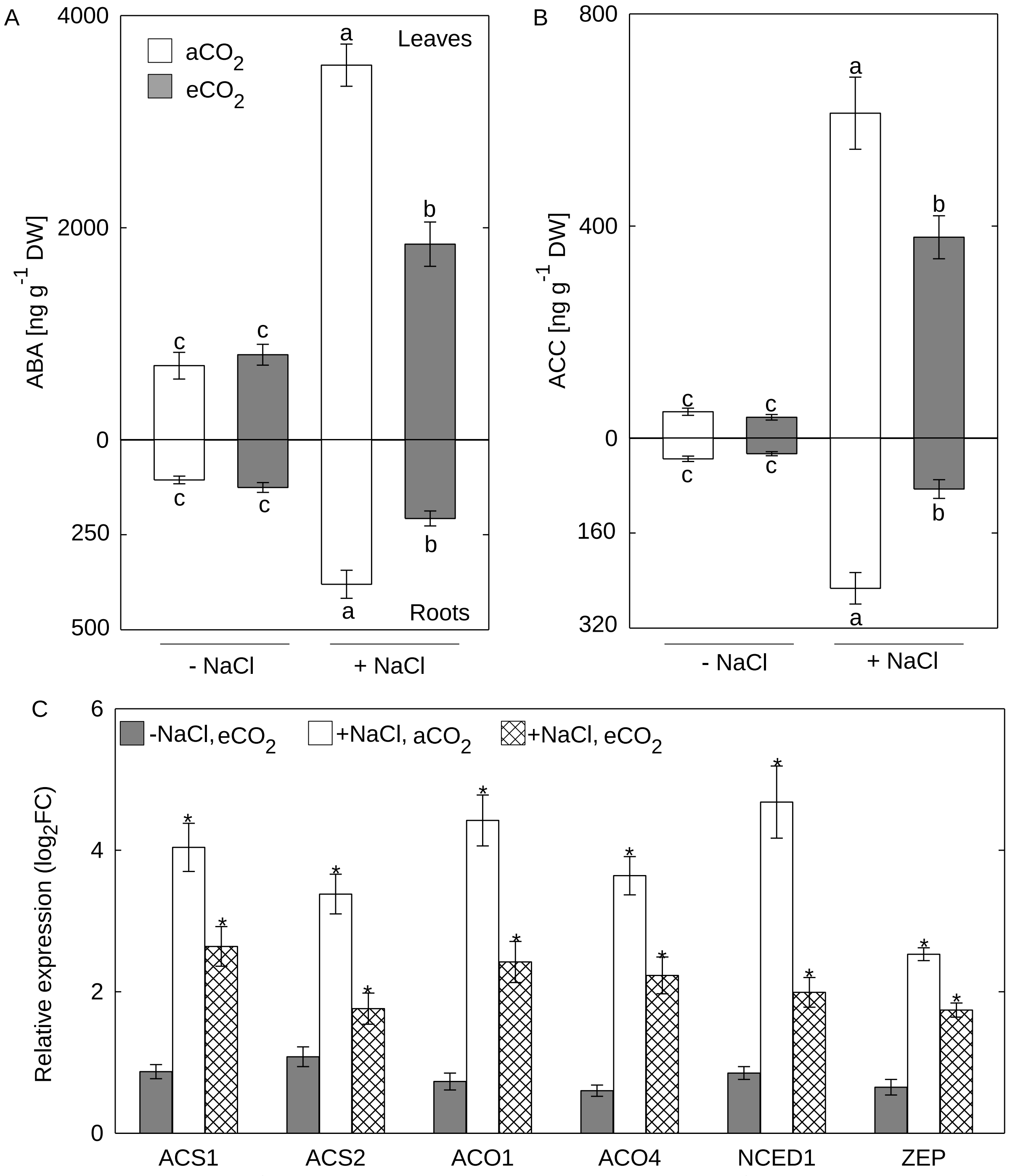

Fig. 3. Concentration of ABA and ACC as well as the expression of genes related with ABA and ethylene biosynthesis in tomato plants treated with NaCl under [aCO$_2$] and [eCO$_2$]. (A) ABA concentrations in leaves and roots. (B) ACC concentrations in leaves and roots. (C) Gene expression in leaves. Bars labelled with the different letters indicate significant differences by Tukey's test at 5% probability. Asterisks indicate values determined by the Student's t-test to be significantly different from control ($P < 0.05$). Data are means ± standard error of three separate experiments, with six replicates for concentration of ABA and ACC, and three replicates for gene expression.

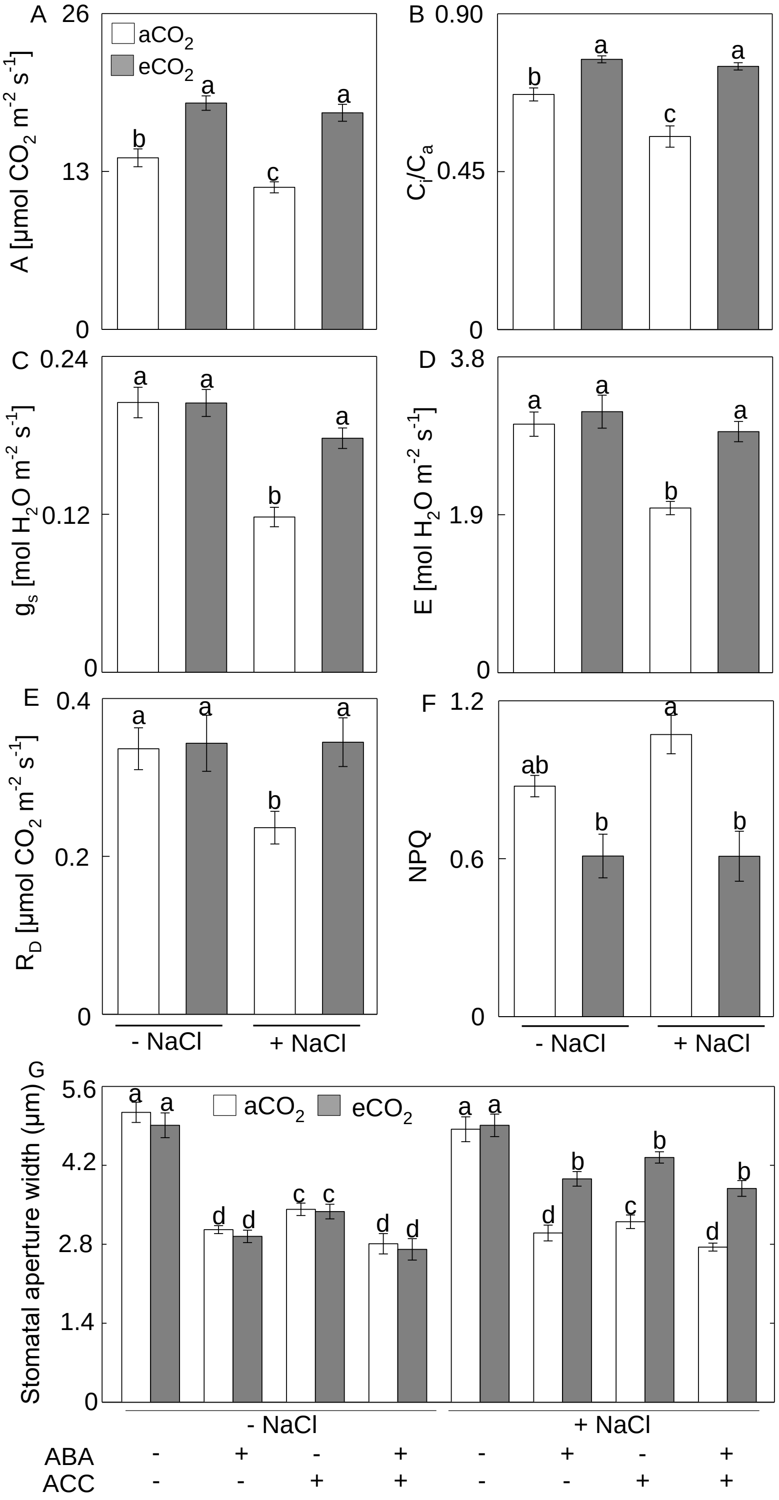

Fig. 4. Physiological parameters observed in leaves of tomato plants treated with NaCl grown under [aCO$_2$] and [eCO$_2$]. (A) Net CO$_2$ assimilation rate. (B) Intercellular-to-atmospheric CO$_2$ ratio. (C) Stomatal conductance. (D) Transpiration rate. (E) Mitochondrial respiration rate in the dark. (F) Non-photochemical quenching coefficient. (G) Stomatal aperture width. Bars labelled with the different letters for A, C$_i$/C$_a$, g$_s$, e, R$_D$ and NPQ indicate significant differences by Tukey's test at 5% probability. Bars labelled with the different letters for stomatal aperture width indicate significant differences by Scott-Knott test at 5% probability. Data are means ± standard error of three separate experiments (n = 100 for stomatal aperture width, and n = 10 for the other parameters).

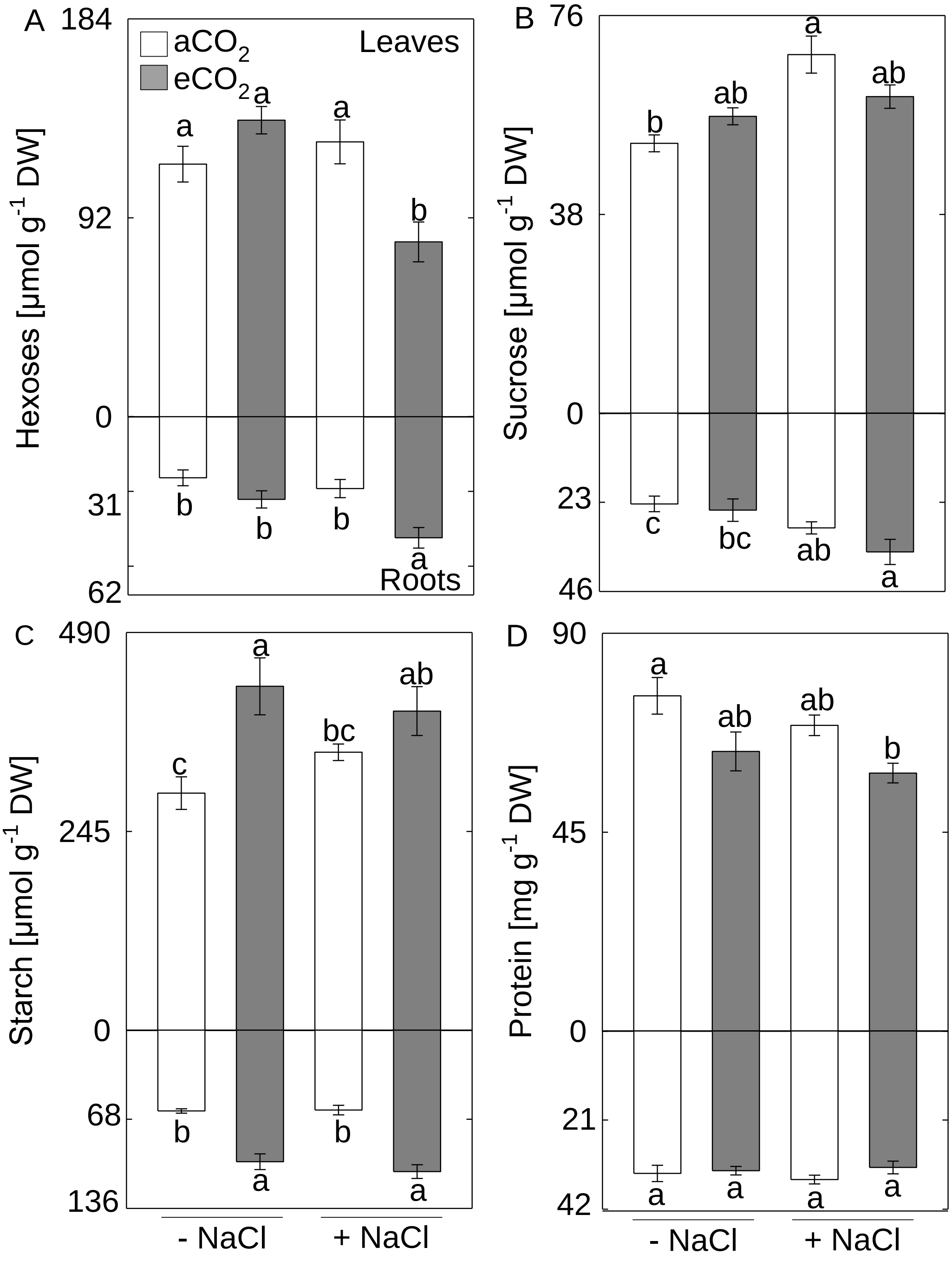

Fig. 5. Levels of hexoses, sucrose, starch and protein in leaves and roots of tomato plants treated with NaCl grown under [aCO2] and [eCO2]. (A) Hexoses. (B) Sucrose. (C) Starch. (D) Protein. Measurements were done using leaves and roots harvested at the end of the light period from plants treated with water (control) or NaCl. Bars labelled with the different letters indicate significant differences by Tukey’s test at 5% probability. Data are means ± standard error of three separate experiments, with six replicates each.

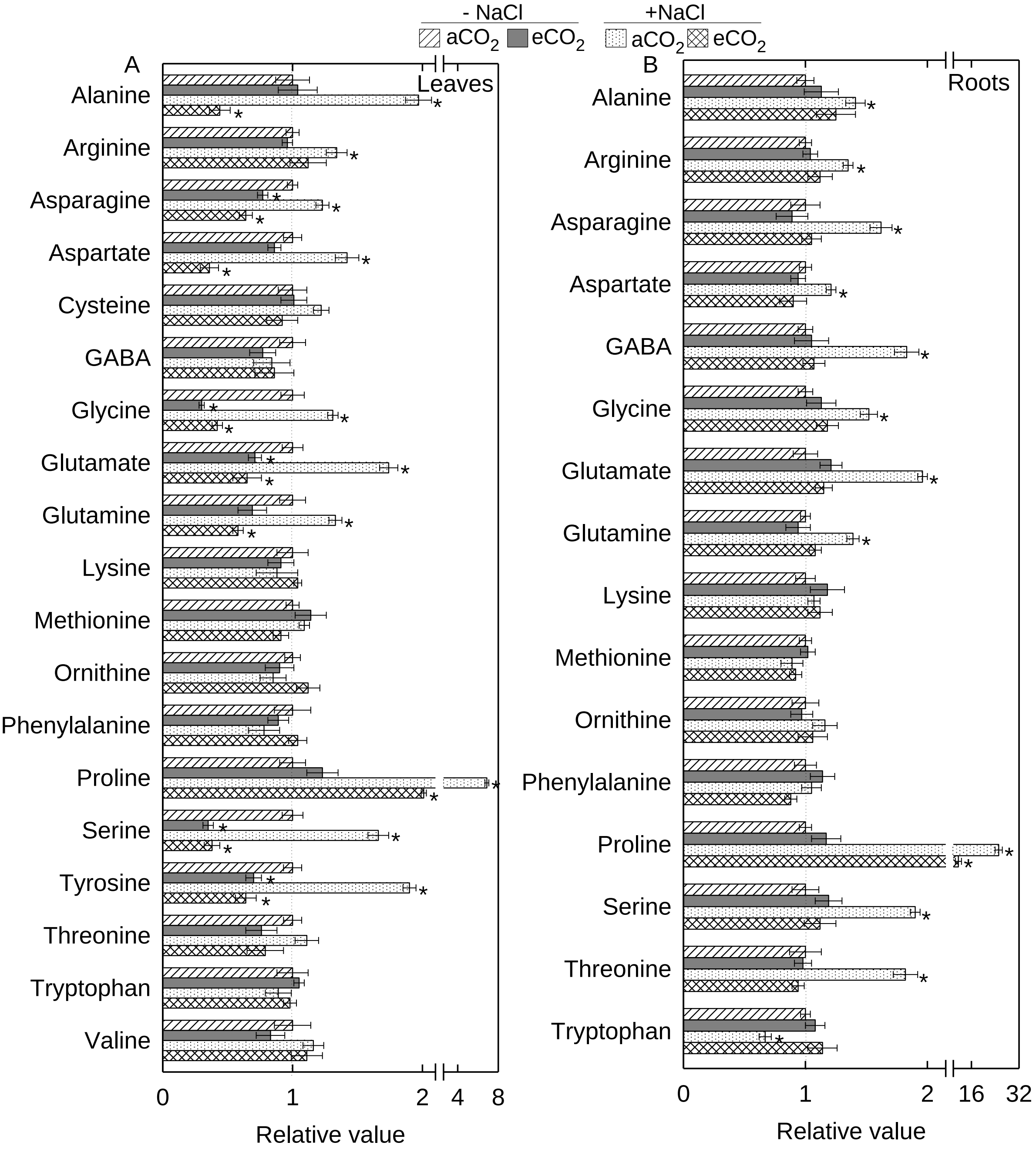

Fig. 6. Changes in the levels of amino acids in leaves and roots of tomato plants treated with NaCl grown under [aCO_2] and [eCO_2]. Measurements were done using (A) leaves and (B) roots harvested at the end of the light period from plants treated with water (control) or NaCl. Data are normalized with respect to mean response calculated for the control treatment (plants grown in soil without NaCl supplementation under [aCO_2]) in each organ. Asterisks indicate values determined by the Student's t-test to be significantly different from control ($P < 0.05$). Data are means ± standard error of three separate experiments, with six replicates each

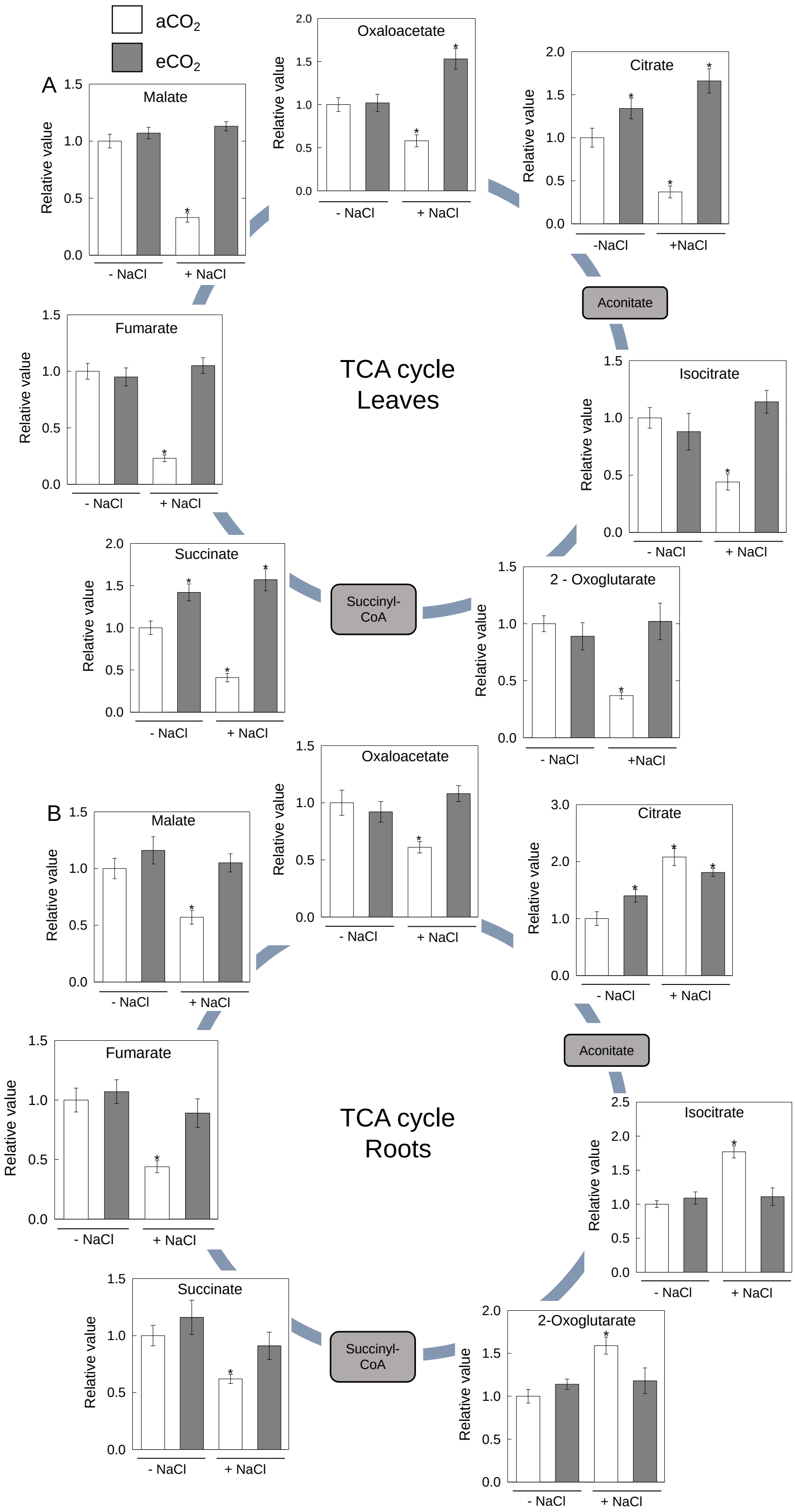

Fig. 7. Changes in the levels of tricarboxylic acid cycle intermediates in leaves and roots of tomato plants treated with NaCl grown under [aCO_2] and [eCO_2]. Metabolites inside grey squares indicate that they were not measured. Measurements were done using leaves and roots harvested at the end of the light period from plants treated with water (control) or NaCl. Data are normalized with respect to mean response calculated for the control treatment (plants grown in soil without NaCl supplementation under [aCO_2]) in each organ. Asterisks indicate values determined by the Student's t-test to be significantly different from control ($P < 0.05$). Data are means ± standard error of three separate experiments, with six replicates each.

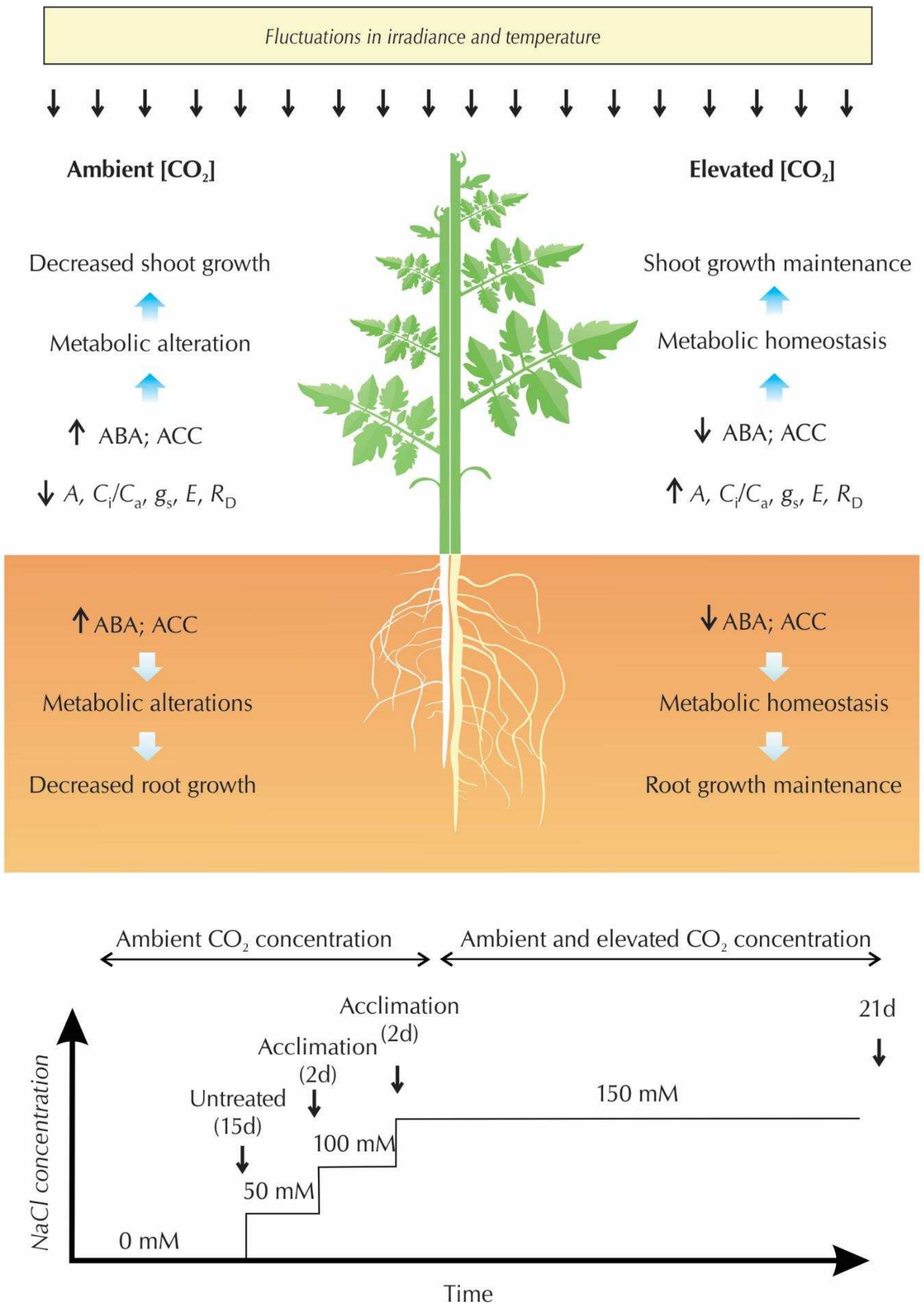

Fig. 8. Overview of tomato plant responses to salt stress under [aCO_2] and [eCO_2]. Diagrams of tomato plants exposure to gradual increasing concentration of NaCl under [aCO_2] (left) and [eCO_2] (right).

Supplementary material

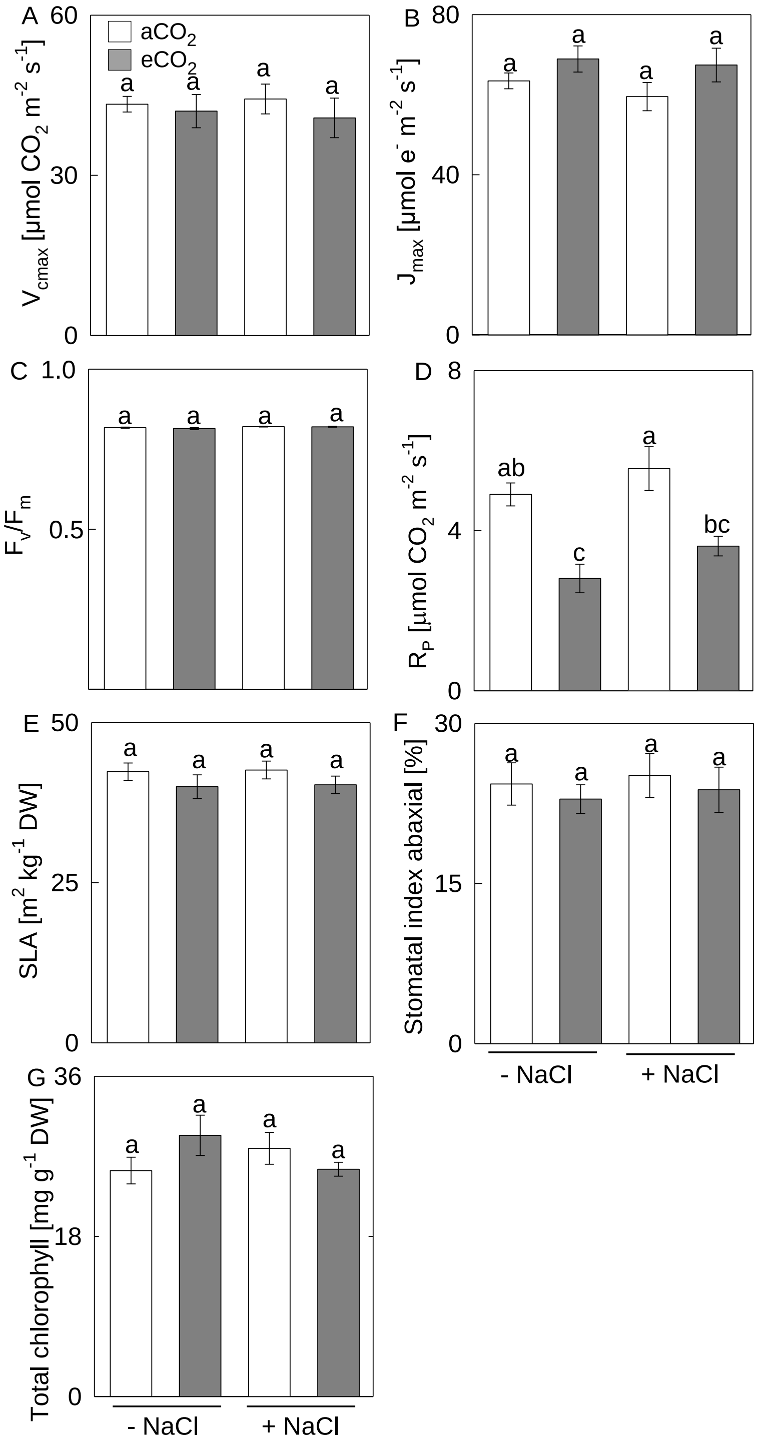

Fig. S1. Physiological parameters observed in leaves of tomato plants treated with NaCl grown under [aCO$_2$] and [eCO$_2$]. (A) Maximum rate of carboxylation. (B) Maximum rate of carboxylation limited by electron transport. (C) Variable-to-maximum fluorescence ratio. (D) Photorespiration. (E) Specific leaf area. (F) Stomatal index. (G) Total chlorophyll concentration. Bars labelled with the different letters indicate significant differences by Tukey's test at 5% probability. Data are means ± standard error of three separate experiments (n = 6 for stomatal index, n = 6 for total chlorophyll concentration and n = 10 for the other parameters).

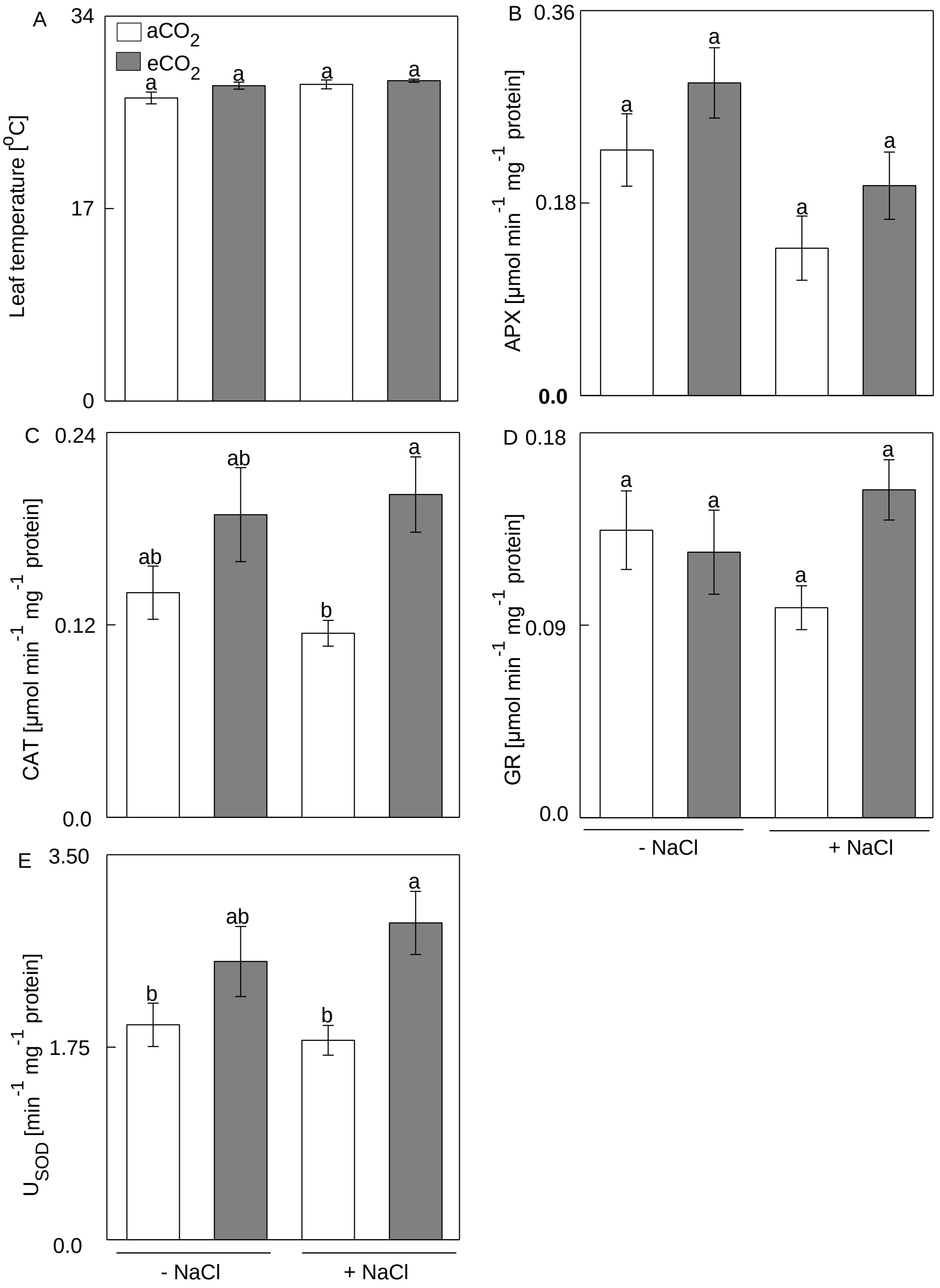

Fig. S2. Leaf temperature and enzyme activities in leaves of tomato plants treated with NaCl grown under [aCO_2] and [eCO_2]. (A) Leaf temperature. (B) Ascorbate peroxidase (APX). (C) Catalase (CAT). (D) Glutathione reductase (GR). (E) Superoxide dismutase (SOD). One unit of SOD was defined as the amount of enzyme that inhibited the reduction of p-nitro-blue tetrazolium by 50 %. Bars labelled with the different letters indicate significant differences by Tukey's test at 5% probability. Data are means ± standard error of three separate experiments (n = 10 for leaf temperature and n = 6 for enzyme activities).

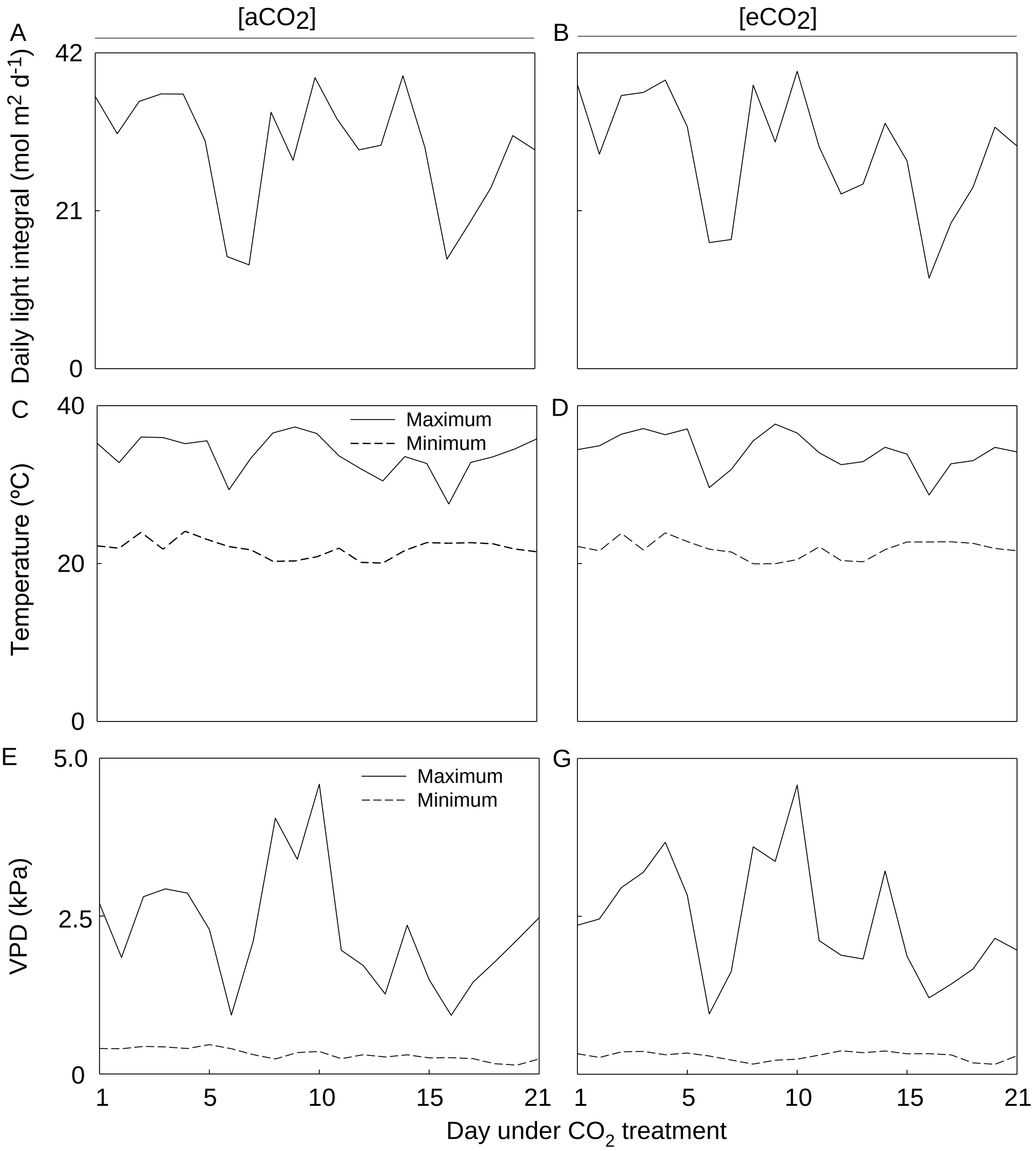

Fig. S3. Fluctuation of daily global irradiance, air temperature and vapour pressure deficit (VPD) during the course of experiment. (A, B) Daily light integral inside the open-top chambers supplemented with ambient ([aCO_2]) and elevated ([eCO_2]) CO_2 concentration. (C, D) Air temperature inside the open-top chambers supplemented with ambient ([aCO_2]) and elevated ([eCO_2]) CO_2 concentration. (E, G) Vapour pressure deficit inside the open-top chambers supplemented with ambient ([aCO_2]) and elevated ([eCO_2]) CO_2 concentration.

Table S1. Primers sequences used for qRT- PCR analysis

Gene	Locus ID	Primer sequence
ACS1	Solyc08g081550	Fwd 5’- TCGTTTCGAAGATTGGATGA-3’
		Rev 5’- CAACAACAACAAATCTAAGCCATT-3’
ACS2	Solyc01g095080	Fwd 5’- CTACGCAGCCACTGTCTTTGAC-3’
		Rev 5’- TGATTCCGACTCTAAATCCTGGTAA-3’
ACO1	Solyc07g049530	Fwd 5’- ACTATCCACCATGTCCTAAGCCCG-3’
		Rev 5’- TCTGTTTGTGCAATTACTCTGTGCAGC-3’
ACO4	Solyc02g081190	Fwd 5’- ACTATCCACCATGTCCTAAGCCCG-3’
		Rev 5’- ACCACACAACAATCACACACA-3’
ZEP	Solyc02g090890	Fwd 5’ -ATGATAGACCGCCAACCTTTAGTT-3’
		Rev 5’-CCATGCATCCCCCTTGAC-3’
NCED	Solyc07g056570	Fwd 5’ -CTGAGAACTTCGTCGTCATTCC-3’
		Rev 5’ -CCCATCTTTCGCGTACTTATCC-3’
Actin	Solyc03g078400	Fwd 5’-GGTCCCTCTATTGTCCACAG-3’
		Rev 5’-TGCATCTCTGGTCCAGTAGGA-3’

Table S2. Relative metabolite level in leaves and roots of tomato plants treated with NaCl under [aCO$_2$] and [eCO$_2$]. Data are normalized with respect to mean response calculated for the control treatment (plants grown in soil without NaCl supplementation under [aCO$_2$]) in each organ. Values set in bold type were determined by the Student's t-test to be significantly different from control ($P < 0.05$). Data are means ± standard error of three separate experiments, with six replicates each.

Metabolite - Leaves	Treatment			
	[aCO$_2$]		[eCO$_2$]	
	- NaCl	+ NaCl	- NaCl	+ NaCl
Fructose	1.00±0.06	0.89±0.07	1.04±0.16	**0.60±0.05**
Glucose	1.00±0.08	0.88±0.11	0.85±0.13	**0.47±0.06**
Glycerate	1.00±0.09	0.79±0.07	0.26±0.03	**0.12±0.02**
3-P-glycerate	1.00±0.10	**0.61±0.05**	**1.21±0.11**	**1.98±0.14**
Lactic acid	1.00±0.08	1.15±0.14	0.95±0.07	0.99±0.10
Myo-inositol	1.00±0.04	**1.63±0.02**	0.89±0.06	1.03±0.12
Alanine	1.00±0.13	**1.97±0.10**	1.04±0.15	**0.44±0.08**
Arginine	1.00±0.05	**1.34±0.08**	0.96±0.04	1.12±0.14
Asparagine	1.00±0.04	**1.23±0.05**	**0.77±0.04**	**0.64±0.05**
Aspartate	1.00±0.07	**1.42±0.09**	0.86±0.05	**0.36±0.07**
Cysteine	1.00±0.11	1.22±0.06	1.01±0.10	0.92±0.12
Glutamate	1.00±0.08	**1.74±0.07**	**0.71±0.05**	**0.65±0.11**
Glutamine	1.00±0.10	**1.33±0.05**	0.69±0.11	**0.58±0.04**
Glycine	1.00±0.09	**1.31±0.04**	**0.30±0.02**	**0.42±0.04**
Lysine	1.00±0.12	0.88±0.16	0.91±0.10	1.04±0.03
Methionine	1.00±0.05	1.09±0.04	1.14±0.12	0.91±0.06
Ornithine	1.00±0.06	0.85±0.10	0.90±0.11	1.12±0.09
Phenylalanine	1.00±0.14	0.78±0.12	0.89±0.08	1.04±0.07
Proline	1.00±0.10	**6.88±0.19**	1.23±0.12	**2.01±0.02**
Serine	1.00±0.08	**1.66±0.08**	**0.35±0.04**	**0.38±0.06**
Threonine	1.00±0.07	1.11±0.09	0.76±0.12	0.79±0.14
Tryptophan	1.00±0.12	0.89±0.10	1.05±0.04	0.98±0.05
Tyrosine	1.00±0.07	**1.90±0.05**	**0.70±0.06**	**0.64±0.08**
Valine	1.00±0.14	1.16±0.08	0.83±0.11	1.11±0.12
Citrate	1.00±0.11	**0.37±0.07**	**1.34±0.12**	**1.66±0.14**
Fumarate	1.00±0.07	**0.23±0.03**	0.95±0.08	1.05±0.07
GABA	1.00±0.10	0.84±0.14	0.77±0.10	0.86±0.15
Isocitrate	1.00±0.09	**0.44±0.07**	0.88±0.16	1.14±0.10
Malate	1.00±0.06	**0.33±0.04**	1.07±0.05	1.13±0.04
2 OG	1.00±0.07	**0.37±0.03**	0.89±0.12	1.02±0.16
Oxaloacetate	1.00±0.08	**0.58±0.07**	1.02±0.10	1.53±0.12
Pyruvate	1.00±0.05	0.78±0.14	0.93±0.07	1.12±0.06
Succinate	1.00±0.08	**0.41±0.05**	**1.42±0.10**	**1.57±0.13**

Metabolite - Roots	Treatment			
	[aCO_2]		[eCO_2]	
	- NaCl	+ NaCl	- NaCl	+ NaCl
Fructose	1.00±0.07	1.26±0.17	1.08±0.09	**1.74±0.14**
Glucose	1.00±0.06	1.16±0.13	1.04±0.11	**1.86±0.10**
3-P-glycerate	1.00±0.10	**2.85±0.16**	1.11±0.10	1.15±0.08
Lactic acid	1.00±0.15	1.17±0.13	1.14±0.10	1.23±0.17
Mannose	1.00±0.04	1.21±0.09	0.92±0.06	1.03±0.09
Alanine	1.00±0.07	**1.41±0.08**	1.13±0.14	1.25±0.16
Arginine	1.00±0.05	**1.35±0.04**	1.04±0.06	1.12±0.10
Asparagine	1.00±0.12	**1.62±0.09**	0.89±0.13	1.05±0.08
Aspartate	1.00±0.05	**1.21±0.04**	0.94±0.06	0.90±0.10
Glutamate	1.00±0.10	**1.96±0.04**	1.21±0.09	1.15±0.07
Glutamine	1.00±0.04	**1.39±0.05**	0.94±0.10	1.08±0.09
Glycine	1.00±0.06	**1.52±0.07**	1.13±0.12	1.18±0.10
Lysine	1.00±0.08	1.07±0.05	1.18±0.14	1.12±0.10
Methionine	1.00±0.05	0.89±0.09	1.02±0.06	0.92±0.05
Ornithine	1.00±0.11	1.16±0.10	0.97±0.09	1.06±0.10
Phenylalanine	1.00±0.09	1.05±0.05	1.14±0.10	0.88±0.08
Proline	1.00±0.05	**25.18±1.02**	1.17±0.12	**11.63±0.07**
Serine	1.00±0.11	**1.90±0.04**	1.19±0.11	1.12±0.06
Threonine	1.00±0.13	**1.82±0.10**	0.98±0.07	0.94±0.12
Tryptophan	1.00±0.04	**0.67±0.05**	1.08±0.08	1.14±0.12
Citrate	1.00±0.12	**2.08±0.15**	**1.40±0.11**	**1.81±0.07**
Fumarate	1.00±0.10	**0.44±0.05**	1.07±0.10	0.89±0.12
GABA	1.00±0.06	**1.83±0.10**	1.05±0.14	1.07±0.08
Isocitrate	1.00±0.05	**1.77±0.09**	1.09±0.09	1.11±0.13
Malate	1.00±0.09	**0.57±0.06**	1.16±0.12	1.05±0.08
2 OG	1.00±0.08	**1.59±0.10**	1.14±0.06	1.18±0.15
Oxalacetate	1.00±0.11	**0.61±0.05**	0.92±0.09	1.08±0.07
Pyruvate	1.00±0.05	1.11±0.09	1.14±0.10	0.98±0.03
Succinate	1.00±0.09	**0.62±0.04**	1.16±0.15	0.91±0.12

CHAPTER 2

Elevated CO_2 enhances growth of tomato plants under salt stress through changes in photosynthesis and primary metabolism, independently of ABA

Abstract

Abscisic acid (ABA) is one of the main regulators of plant tolerance to salt stress conditions. In addition, the elevated concentration of CO_2 (e[CO_2]) is able to stimulate plant growth and mitigate the effects of salinity in plants. However, physiological mechanisms involving e[CO_2] and endogenous levels of ABA under salinity conditions remain poorly understood. In this study, we investigated how carbon assimilation and biomass accumulation are affected by e[CO_2] in wild type tomato (Solanum lycopersicum L.) cv. Micro-Tom and in mutants producing low (notabilis, not) and high (NCED) endogenous levels of ABA. These responses were also investigated under salinity stress (75 mM NaCl in soil). Growth of ABA-deficient (not) plants was strongly reduced compared to MT and NCED plants in both [CO_2], mainly when not plants were submitted to treatment with NaCl. However, e[CO_2] increased total biomass and leaf area of MT, not and NCED plants under saline stress compared to ambient [CO_2] (a[CO_2]). Moreover, NCED plants under e[CO_2] showed higher growth performance compared to WT and not mutant in non-saline and saline conditions. e[CO_2] stimulated gas exchange accompanied by low photorespiration rates in all genotypes under salinity. In addition, e[CO_2] was able to improve the water status of MT and not plants treated with NaCl in relation to a[CO_2]. e[CO_2] also induced changes in primary metabolism of MT, not and NCED plants, which was associated with an increase in dark respiration, especially of MT and not mutant under saline stress. Collectively, our results suggest that ABA is essential to support plant growth under saline stress, but e[CO_2] can mitigate the effects of salt stress through increasing photosynthesis and adjustments in primary metabolism by mechanisms independent of ABA concentration.

Keywords: Hormones, Micro-Tom, notabilis, Primary metabolism, Salt stress, Solanum lycopersicum L.

1 Introduction

The elevation of the CO_2 concentration (e[CO_2]) in the atmosphere is one of the main problems contributing to global warming and it is estimated that, with the continuous advance of anthropogenic activities, the [CO_2] will be around 800 ppm by the end of this century (IPCC, 2014). Studies show that e[CO_2] has positive impacts on plant growth, often associated with increases in the net CO_2 assimilation rates in plants with C3 metabolism (Kaiser et al., 2017; Xu et al., 2015). e[CO_2] also affects cell division and cell expansion as well as hormone metabolism (Gupta et al., 2005; Kiba et al., 2019; Masle, 2000). Still in the scenario of climate change, inadequate soil cultivation and the use of saline water for irrigation causes soil salinity, reducing the productivity of agricultural crops. In this respect, plant growth is impaired in saline soils due to unbalanced water and nutrient absorption, which induces ionic toxicity and metabolism disorders (Munns e Tester, 2008; Ismail e Horie, 2017; Wang et al., 2017). A range of studies show that salinity damage can be mitigated in plants grown under e[CO_2] (Geissler et al., 2010; Li et al., 2013; Piñero et al., 2014). The stimulus of plant growth provided by e[CO_2] under salinity condition involves increase in photosynthetic rate, nutrient absorption and assimilation, and reduction of oxidative stress (Pérez-López et al., 2015, 2009; Yu et al., 2015). In addition, plants under e[CO_2] tend to partially reduce stomatal conductance (g_S), which may increase water use efficiency and reduce salt transport to leaves (Del Amor, 2013; Gao et al., 2015; Yi et al., 2015). Despite the well-known role of e[CO_2] in the mitigation of saline stress, its interrelationships in the control of hormonal regulatory pathways remains hitherto unclear.

The acclimation responses of plants to saline stress are controlled mainly by abscisic acid (ABA) concentrations (Suzuki et al., 2016; Zhang et al., 2016). The 9-cis-epoxycarotenoid dioxygensase (NCED) plays a key role in ABA biosynthesis during stress conditions such as water deficit and salinity (Huang et al., 2018). The accumulation of ABA in the leaves induces rapid stomatal closure and modulates plant growth through gene expression (Kuluev et al., 2017). The reduction of g_S, induced by ABA, in saline stress condition under ambient [CO_2] (a[CO_2]), is associated with the decrease of photosynthetic rate and consequently, with the inhibition of plant growth (Hayat et al., 2011; Piñero et al., 2014). Under non stressed conditions, plants grown under e[CO_2] can reduce the expression of ABA biosynthesis genes and the total ABA levels in tissues (Teng et al., 2006; Wei et al., 2013). Other results about the co-action between ABA and e[CO_2] under non stressing conditions indicates that ABA participates, at least partially, in the stomatal closure induced by e[CO_2] (Chater et al., 2015). Moreover, there is a convergence point between the signal transduction pathways induced by e[CO_2] and ABA that promotes stomatal closure (Engineer et al., 2016; Hsu et al., 2018; Merilo et al., 2013). In saline stress, ABA levels were reduced in Capsicum annuum roots submitted to e[CO_2]. This reduction in root ABA concentration was associated with improved stomatal control and, consequently, the tolerance of plants to salinity (Piñero et al., 2014). In this context, the effect of ABA and e[CO_2] on the rapid reduction of conductance could be useful to ensure the development of plants under saline stress. Nevertheless, tomato plants (Solanum lycopersicum L. cv. Santa Clara) grown under e[CO_2] with fluctuating irradiance and temperature in soil with saline irrigation showed recovery of biomass and metabolic homeostasis, which was accompanied by maintenance of g_S under e[CO_2] (Brito et al., 2020). These data were associated with the reduction of NCED expression which resulted in lower levels of ABA allowing the best stomatal opening in saline stress and e[CO_2] (Brito et al., 2020). In this context, the flexibility in stomatal response with respect to e[CO_2] leads to new questions about the ABA role in responses to saline stress and variations in environmental conditions.

Tomato plants with manipulations in the ABA biosynthesis pathway are relevant strategies for understanding the role of this hormone in saline stress (Martínez-Andújar et al., 2020a; Mulholland et al., 2003). In order to investigate whether plants with alteration in ABA biosynthesis present different tolerance strategies to salt stress under a[CO_2] and e[CO_2], we analyzed physiological parameters and growth of mutants with low (notabilis, not) and high (NCED) endogenous ABA concentration, all in the same background (cv. Micro-Tom). The present study shows that ABA biosynthesis is essential for the development of plants under saline stress, but the induction of plant growth by e[CO_2] in salinity is dependent on alterations in photosynthetic capacity, maintenance of water status and changes in carbon metabolism.

2 Materials and methods

2.1 Plant material and experimental design

Seeds of tomato plants (Solanum lycopersicum L.) cv. Micro-Tom (MT) and near isogenic lines notabilis (not) and NCED in the same background, were obtained as previously described by Carvalho et al. (2011) and Robledo et al. (2020). Seeds were surface-sterilized with 5% sodium hypochlorite for 10 min and then washed with running distilled water and subsequently sowed in trays containing commercial substrate (Tropstrato HT, Mogi Mirim, Brazil). Around 7 days after germination, seedlings were transferred to plastic pots with a 1.2 L capacity, contained the commercial substrate (Tropstrato HT) supplemented with 1 g L^{-1} 10:10:10 NPK and 4 g L^{-1} dolomite limestone ($MgCO_3$ +$CaCO_3$). Seedlings were established under a[CO_2] in greenhouse at the Universidade Federal de Viçosa (20º45’ S, 42º 15’ W, 650 m altitude) in southeastern Brazil. When the third true leaves appeared, seedlings were watered with deionized water (control) or 75 mM NaCl solution and were placed in open-top chamber (4.2 m diameter and 2.4 m high) in a random arrangement with 42 pots per chamber under a[CO_2] (400 ± 20 μmol mol^{-1}) or e[CO_2] (750 ± 30 μmol mol^{-1}). Plants in the open-top chambers were watered at 2-days intervals with deionized water (control) or 75 mM NaCl solution for 21 days, under natural photoperiod. Compressed gas cylinder was used to constantly enrich the atmosphere with CO_2 in the open top chamber. The [CO_2] was monitored by infrared gas analyzer (Li-840, LI-COR, Lincoln, NE, USA). Four independent experiments were carried out in the months of December 2018 and August 2019 with monitoring of temperature, relative humidity and photosynthetic photon flux density (PPFD), recorded every 30 min by sensors linked to data logger (Li-1400, Li-COR, Lincoln, NE, USA). The vapour-pressure deficit (VPD), daily light integral (DLI) and air temperature were averaged for both open top chambers during the period of the experiments (Fig S3).

2.2 Analysis of plant growth

At the end of each experiment (at 21 days of CO_2 supplementation in open top chambers), the height of the plants was measured from ground level to the upper apical node. The number of leaves longer than 1 cm were counted and roots, stem and leaves were separated and the total leaf area was determined by area meter (Li-Cor 3100, LI-COR, Lincoln, NE, USA). Roots were washed with water over a 0.5 mm mesh sieve (Ribeiro et al., 2016). Subsequently, the leaves, stems and roots were oven dried at 65 °C until constant mass for determining dry weight (DW). The relative growth rate (RGR) was estimated as described by Hunt (1990): RGR = $[(\ln DW_2 - \ln DW_1)/(t_2 - t_1)]$. Where DW_1 and DW_2 are total plant dry weights at respective time t_1 (plants harvested at day 1) and t_2 (plants harvested at day 21) on open-top chambers. The relative water content (RWC) was performed by collecting leaves at pre-dawn and at midday and was calculated from the equation: RWC = $[(FW - DW)/(TW - DW)] \times 100$. Where FW is leaf fresh weight, DW is leaf dry weight and TW is leaf turgid weight achieved by floating leaves on petri dish with distilled water for 12 h (Ribeiro et al., 2009).

2.3 Chlorophyll fluorescence and gas exchanges measurements

The net carbon assimilation rate (A), the stomatal conductance (g_s), internal CO_2 concentration (C_i), ratio between the internal and external CO_2 concentration (C_i /C_a) were determined simultaneously with chlorophyll a fluorescence parameters using an infrared gas analyzer (LI-6400XT, LI-COR, Lincoln, NE, USA) equipped with an integrated fluorescence chamber (LI-6400-40; LI-COR Lincoln, NE, USA). Gas exchange was measured between 9:00 and 11:00 h, under photosynthetically active radiation equivalent to environment (1000 ± 100 µmol m^{-2} s^{-1}). The reference CO_2 concentration was 400 µmol CO_2 mol^{-1} air for plants under a[CO_2] and 750 µmol CO_2 mol^{-1} air for plants under e[CO_2]. The initial fluorescence (F_0) was obtained by lighting leaves adapted to the dark for two hours, using low intensity modulated red light (0.03 µmol photons m^{-2} s^{-1}). Then, a pulse of 0.8 s of saturated actinic light (8000 µmol photons m^{-2} s^{-1}). It was applied to determine the maximum fluorescence (F_m). The maximum photochemical efficiency of photosystem II (FSII) was calculated: $F_v/F_m = [(F_m - F_0)/F_m)]$ and the non-photochemical extinction coefficient (NPQ) were estimated, as described by Martins et al. (2014). The dark respiration (R_N) was quantified using the same equipment described above, two hours after the end of the light period, as described by Brito et al. (2020).

2.4 Metabolite assays

Leaves and roots were collected at the end of the light period and immediately frozen in liquid nitrogen and stored at a temperature of -80 ºC to continue the analyses. A fraction of the lyophilized samples (± 20 mg) was used for ethanolic extraction, as described by Geigenberger et al. (1996). Hexoses (glucose + fructose) and sucrose were determined using a continuous enzymatic substrate assay, as described by Fernie et al. (2001), from the extracted supernatant. Amino acids levels were measured as previously described by Cross et al. (2006). For the quantification of starch and proteins, the precipitated residue from ethanol extraction was solubilized in 0.1 M NaOH, at 95 ºC. Afterwards, for determining starch levels the acetic acid (1 M) was added and the starch content was determined as glucose equivalents, according to Fernie et al. (2001). Protein levels were quantified using the Bradford technique (Bradford, 1976) using the Bio-Rad Bradford reagent (Bio-Rad Laboratories). Total chlorophyll levels were extracted in 80% acetone from the 0.5 cm^2 leaf disc section and quantified as described by Porra et al. (1989). Proline levels in leaves and roots were measured using 50 µL aliquot of the ethanolic extract and reacted with the reaction medium containing 20% (v/v) ethanol and 1% (w/v) ninhydrin diluted in 60% (v/v) glacial acetic acid. To start the reaction, the samples were shaken and heated to 95 ºC for 20 minutes in the dark (Carillo et al., 2008).

2.5 Quantification of Na^+, K^+ and Total N

The levels of Na^+ and K^+ in leaves were determined following the protocol described by Munns et al. (2010) was carried out. Samples of dried leaves were crushed using a Wiley mill (CINELAB CE-430; 8 blades, 1,725 rpm, 20 mesh size) and ± 0.5 g of leaves were digest with $HNO_3/HClO_4$ (2:1, v/v) and subsequently, the analysis was performed on an induced plasma optical emission spectrophotometer (ICP-OES, Perkin Elmer Optima 3000 XL). Total nitrogen was extracted by sulfuric digestion and measured by Kjeldahl method (Baethgen and Alley, 1989).

2.6 Stomatal index and stomatal pore aperture

Portions of five central leaflets of fully expanded leaves were collected at the end of the light period and diaphanized in 95% (v/v) methanol for 48 h, followed by 100% lactic acid at 95 ºC in a water bath until they were totally translucent (Zsögön et al., 2015). The leaflets were arranged on glass slides, and the images of the abaxial epidermis were obtained under a light microscope (AX-70 TRF, Olympus Optical, Tokyo, Japan), coupled to the digital camera (Zeiss AxioCam HRc, Göttinger, Germany). The stomatal index was performed by counting the stomata in 3 images 0.58 mm^2 for each replicate and the stomatal pore aperture was quantified by measuring 30 stomata per image. The microscope images were captured on a computer with Axion Vision software and stored for measurements in the Image-Pro® Plus (version 4.5).

2.7 Experimental design and statistical analysis

The experiments were performed in a completely randomized design. For the metabolites assays, 6 independent replicates of leaves and roots collected from 3 experiments were used. To the determination of mineral elements independent samples of 4 leaves from 3 experiments were used. In the gas exchange and chlorophyll fluorescence experiments, the average of two experiments was carried out with sampling of 6 independent plants per treatment. Data were subjected to three-way analysis of variance (ANOVA, $P \leq 0.05$) and the treatment means were compared by the Tukey's test ($P \leq 0.05$) using the SPSS statistical package software.

3 Results

3.1 Elevated [CO_2] stimulates plant growth, regardless of ABA levels in tissues

Plants with low ABA concentrations (not) had limitations in the growth and maintenance of water status in relation to MT and NCED plants, regardless of the levels of atmospheric CO_2 (Fig. 1A). NaCl supplementation reduced the performance of tomato plants in most of analyzed morphological parameters. The interaction analysis indicates that the leaf area (LA) has only a significant effect between the genotype and the salinity treatment (Table

1). Plants with reduced (not) and increased (NCED) expression of ABA biosynthesis showed reductions in LA under salinity conditions and a[CO_2], while e[CO_2] stimulated gain in LA only in plants MT and NCED, in -NaCl and +NaCl treatments (Fig. 1B). There was a reduction in the LA of +NaCl plants in a[CO_2], while e[CO_2] allowed higher LA in saline conditions (P = 0,0001). There was no significant interaction between the factors in plant height (Table 1). However, the height of +NaCl plants under a[CO_2] was lower than those of the control (-NaCl), for all observed genotypes (Fig. 1C). On the other hand, e[CO_2] led to an increase in height for all plants treated with NaCl (Fig. 1C).

The analysis of the mass parameters of leaves, stems and roots showed that there is an interaction effect between [CO_2] x genotypes, and for the stem there was a significant interaction between treatments x genotypes (Table 1). MT +NaCl plants showed reductions in the mass of leaves (34 %), stems (48 %) and roots (43 %) under a[CO_2], compared to -NaCl plants (Fig. 1 D-F). Once subjected to e[CO_2] there was recovery in the mass of leaves (54 %), stems (100 %) and roots (72 %), compared to -NaCl plants under a[CO_2] (Fig. 1F). In not plants the development of leaf, stems and roots was less in relation to the MT and NCED genotypes, showing no significant differences when evaluated by triple factorial statistics (Fig. 1 D-F). In contrast, when analyzed in one-way ANOVA, followed by Tukey's test ($p < 0.05$), it is observed that there are statistical differences for all parameters of leaf (55 %; P = 0,0001), stems (99%; P = 0,0001) and roots mass (64 %; P = 0,0001). In this way, not plants with drastic reductions in the mass of leaves, stems and roots under saline stress, restored, at least in part, their mass under e[CO2] (Fig. 1A). Leaves of NCED plants were not affected by +NaCl under a[CO_2], however an increase in leaf dry mass was observed when subjected to e[CO_2] under non-saline conditions (Fig. 1D). On the other hand, dry mass of the stems and roots of NCED mutants were significantly increased by e[CO_2] for both -NaCl and + NaCl treatments (Fig. 1E-F). The interaction analysis indicates that the relative growth rate (RGR) is dependent on genotype and treatments with NaCl (Table 1). The RGR of MT and not plants was affected by salt stress in a[CO_2], however e[CO_2] stimulated the growth of all genotypes, including plants treated with NaCl (Fig. 1G).

3.2 Na^+ foliar absorption is not modulated by ABA and CO_2 levels

The interaction analysis indicates that the absorption of Na^+ is entirely dependent on genotype and [CO_2] (Table 1). Under salt treatment (+NaCl), not plants showed higher levels of Na^+ in leaves than MT and NCED plants under a[CO_2] (Fig. 2A). On the other hand, under salt stress and e[CO_2] all genotypes showed high levels of Na^+ in the leaves (Fig. 2A) indicating that the concentration of Na^+ in the leaves is independent on the transpiration rate. K^+ levels were not affected by salinity under a[CO_2] conditions for all evaluated genotypes (Table 1). In contrast, under e[CO_2], only not mutant showed higher levels of K^+ in leaves compared to other genotypes under non-saline treatment (Fig. 2B). Moreover, the supplementation with NaCl did not inhibit the transport of K^+ by the leaves in both [CO_2] (Fig. 2B). Additionally, salt stress increased the Na:K ratio for the three genotypes evaluated without influence of [CO_2] (Fig. 2C).

3.3 Photosynthetic parameters

Treatment with NaCl provoked decreases in A in MT and not plants grown under a[CO_2] (Fig. 3A). NCED mutants showed lower A values than MT(-NaCl) under a[CO_2], but were not affected by treatment with NaCl (Fig. 3A). Increases in A in plants under e[CO_2] were observed for all genotypes in -NaCl and +NaCl treatments (Fig. 3A). e[CO_2] resulted in high levels of C_i for all genotypes evaluated under -NaCl and +NaCl conditions compared to a[CO_2] (Fig. 3B). Under a[CO_2], NaCl reduced g_S and E in MT and not plants, but g_S was not affect by salt stress in NCED plants (Fig. 3C; D), indicating that the stomatal regulation under these conditions may be influenced by other factors in addition to ABA. Nevertheless, partial recovery of g_S and increases in E in not plants when grown in e[CO_2] was observed (Fig. 3C; D).

Factorial analysis indicates that dark respiration (R_N) was significantly altered by e[CO_2] (Table 1; Fig. 3E). e[CO_2] stimulated an increase in R_N in +NaCl MT and not plants under -NaCl and +NaCl treatments (Fig. 3E). In general, saline stress did not influence R_P in MT and not plants under a[CO_2], but R_P was increased in NCED mutants in a[CO_2] (Fig. 3F). In contrast, e[CO_2] led to a decrease in R_P in all genotypes grown under control and saline conditions (Fig. 3F). R_P values for NCED plants treated with NaCl under a[CO_2] may be associated with increases in ETR under these conditions (Fig. 3G). Under a[CO_2], not plants treated with NaCl exhibited reductions in F_v/F_m compared to other genotypes under the same conditions (Fig. 3H). In contrast, there was an increase of F_v/F_m in not plants treated with NaCl grown under e[CO2], when compared with not plants under a[CO_2]. Changes in F_v/F_m were not observed for plants MT and NCED treated with NaCl under e[CO_2] (Fig. 3H).

3.4 The water status impaired by the low levels of ABA is minimalized under e[CO2].

There were no differences in stomatal index in any genotype in relation to [CO_2] under treatments of -NaCl and + NaCl (Fig. 4A). Under non-saline and saline stress ABA-deficient mutant have a higher stomatal index compared to MT and NCED plants in both a[CO_2] and e[CO_2] (Fig. 4A). Salinity reduced stomatal opening for not, MT and NCED genotypes under both [CO_2] (Fig. 4B). However, the stomatal opening of not plants was greater than MT and NCED plants under a[CO_2] and e[CO_2], regardless of treatment with NaCl (Fig. 4B). Plants under salt stress had lower RWC in all genotypes at pre-dawn under a[CO_2] and e[CO_2] (Fig. 4C). However, under e[CO_2] there was an increase in RWC in MT +NaCl and not -NaCl plants (Fig. 4C). Salinity intensified the reduction of RWC in MT, not and NCED genotypes under a[CO_2] and e[CO_2] (Fig. 4D). However, under a[CO_2] the not plants in control and NaCl conditions showed lower RWC at midday compared to MT and NCED plants under the same conditions (Fig. 4D). In addition, it is noteworthy that MT and not plants grown under control (-NaCl) and salinity conditions were able to increase RWC at midday under e[CO_2] (Fig. 4D).

3.5 ABA and CO_2 modify the metabolite production ratio in plants under salt stress.

In leaves there was a notable difference in the total content of free hexoses between the genotypes (Fig. 5A). The not plants showed low levels of hexoses in relation to MT and NCED plants grown under control and saline conditions under both a[CO_2] and e[CO_2] (Fig. 5A). However, MT and NCED plants under e[CO_2] and control and +NaCl treatments increased the hexose content in leaves in relation to a[CO_2] (Fig. 5A). By contrast, under a[CO_2], roots of NCED plants grown under -NaCl and +NaCl conditions showed higher concentration of hexoses compared to the MT and not genotypes in non-saline and saline conditions (Fig. 5A). Under e[CO_2], hexoses levels remained stable in roots of NCED plants (-NaCl and +NaCl) in relation to a[CO_2] (Fig. 5A). In addition, concentrations of hexoses increased in roots of MT plants irrigated with non-saline and saline solution under e[CO_2] compared to a[CO_2] (Fig. 5A). An increase in hexose was observed in the not roots (-NaCl) under e[CO_2] in relation to its control under a[CO_2] (Fig. 5A).

Leaves of not plants (-NaCl and +NaCl) showed higher sucrose concentration under both a[CO_2] and e[CO_2] compared to MT and NCED plants (Fig. 5B). In addition, sucrose was reduced by 15 % for not -NaCl and 12 % for not +NaCl when subjected to e[CO_2] (Fig. 5B). There were no changes in concentrations of sucrose in leaves of MT and NCED plants under both [CO_2]. Under a[CO_2], the sucrose concentration was higher in roots of NCED (+ NaCl and -NaCl) and MT (+NaCl) plants compared to MT (-NaCl) and not plants in non-saline and saline treatments (Fig. 5B). Moreover, concentrations of sucrose in roots of plants MT and NCED treated with NaCl increased under e[CO_2] compared to a[CO_2] (Fig. 5B). Under e[CO_2], concentrations of sucrose increased in roots of not plants grown under control and saline conditions, when compared with not plants under a[CO_2] (Fig. 5B). Regarding starch, saline stress impaired its biosynthesis in all genotypes, under a[CO_2], however, considerable increases were observed in MT and not plants under -NaCl and +NaCl conditions, as well as in NCED plants treated with NaCl under e[CO_2] (Fig. 5C). There were no significant changes in starch levels in roots of not plants in relation to both [CO_2] and non-saline and saline conditions (Fig. 5C). Under a[CO_2], roots of NCED untreated with NaCl showed higher levels of starch compared to +NaCl NCED plants and the MT and not genotypes under non-saline and saline treatments (Fig. 5C). On the other hand, e[CO_2] induced an increase of starch in MT roots untreated with NaCl and in NCED roots under non-saline and saline conditions (Fig. 5C).

Under control conditions (-NaCl), there were no changes in leaf amino acid levels of the 3 genotypes in a[CO_2] (Fig. 6A), while under e[CO_2] higher values of total amino acids were observed in MT leaves under non-saline conditions (Fig. 6A). By contrast, saline treatment resulted in leaves with remarkable accumulation of amino acids for MT, not and NCED plants under a[CO_2], while a significant reduction in amino acids was observed in all genotypes treated with NaCl under e[CO_2] (Fig. 6A). Under a[CO_2], roots of NCED plants untreated and treated with NaCl showed lower amino concentration compared to MT plants grown under control conditions and not mutant untreated and treated with NaCl (Fig. 6A). Saline stress did not alter the concentrations of amino acids in roots of the genotypes grown under a[CO_2] (Fig. 6A). Moreover, e[CO_2] did not influence amino acid levels as observed in a[CO_2] for MT and NCED roots, however there was a reduction in concentrations of amino acids in not plants in both non-saline and saline conditions (Fig. 6A). Plants irrigated with saline solution showed high proline accumulation in leaves and roots of all genotypes under a[CO_2] (Fig. 6B). Under e[CO_2] and salt stress, the increase in leaf proline was lower for MT and not plants compared to plants under a[CO_2] (Fig. 6B). Among all genotypes grown in a[CO_2], no differences were observed in protein concentration in leaves of plants grown under non-saline and saline conditions (Fig. 6C). Under a[CO_2], the roots of not mutants grown under control and saline conditions, showed higher concentrations of proteins than the other genotypes (Fig. 6C). In e[CO_2], not mutants accumulated more proteins in leaves in relation to the MT and NCED plants grown under non-saline and saline conditions. Salinity reduced the total N concentration only in not plants treated with NaCl under a[CO_2] (Fig. 6D). Furthermore, no change in total N concentration was found in other genotypes and treatments (Fig. 6D). Regarding the total chlorophyll concentration, salinity affected its concentration in MT plants grown under a[CO_2] (Fig. 6E). NCED plants showed lower chlorophyll values under non-saline and a[CO_2] conditions compared to MT and not genotypes under control treatment (Fig. 6E). However, the concentration of chlorophyll in NCED plants under control condition (-NaCl) was increased under e[CO_2] in relation to a[CO_2] (Fig. 6E).

4 Discussion

Plant responses to environmental stimuli revealed the physiological role of ABA as a central regulatory mechanism for growth acclimation processes under abiotic stress (Vishwakarma et al., 2017). Moreover, several studies have shown that the inhibitory effect of salinity on plant growth is mitigated in e[CO_2] due to the C input which promotes an increase in photosynthesis and a reduction g_S, thereby increasing water use efficiency (Piñero et al., 2014; Zaghdoud et al., 2016). Stomatal closure induced by e[CO_2] was associated with the accumulation of ABA in the xylem and leaves (Fang et al., 2019; Wei et al., 2020). Recently, Brito et al. (2020) pointed out that e[CO_2] restored the growth of tomato plants cv. Santa Clara under saline stress by increasing of photosynthesis, maintaining metabolic homeostasis and stomatal opening. The growth restoration of tomato plants treated with NaCl grown under e[CO_2] was associated with a reduction in concentrations of ABA and ACC. However, the role of ABA in regulating the plant development under e[CO_2] remains poorly defined. The results of the present study have revealed that ABA is required for plant development under non-saline and saline conditions, but it is not directly necessary for e[CO_2]-induced growth.

The relationship between ABA and plant growth seems to be very restricted to its endogenous concentration (Humplík et al., 2017). Here, we provide evidence that endogenous ABA is required for establishment of tomato plants under non-saline and saline conditions (Fig. 1). ABA plays an essential role in natural and stress conditions as it leads to the maintenance of water status and leaf expansion (Martin et al., 2017; Thompson et al., 2007a). This was visible in not plants with compromised biomass accumulation compared to MT (Fig. 1). On the other hand, previous studies have shown that increased NCED transcript levels resulted in increased ABA biosynthesis, which can impair plant growth (Luo et al., 2014; Tung et al., 2008). However, the growth of tomato plants with overexpression of NCED was not inhibited by salt stress under a[CO_2], since these plants presented growth parameters similar to MT in the control (-NaCl) under a[CO_2] (Fig. 1). When NCED plants treated with NaCl were grown under e[CO_2] they showed increases in leaf area, height and dry mass of leaves and stems compared to MT control plants (-NaCl) (Fig. 1). The ABA content in leaves and xylem of tomato plants with overexpression of NCED1 (sp12 line) is slightly higher compared to cv. Ailsa Craig (Martínez-Andújar et al., 2020b; Thompson et al., 2007b). The small increment in ABA content of NCED plants in relation to MT may explain the small variation in relation to their growth under a[CO_2]. NCED and MT plants reduced shoot and root biomass when subjected to salinity (Fig. 1) indicating that high ABA levels is necessary for plants to maintain their growth in salinity conditions (Martínez-Andújar et al., 2020).

Here, all genotypes showed partial recovery from the effects of salinity in e[CO_2] (Fig. 1). Tomato plants WT and mutants grown under e[CO_2] showed an increase in A in line with high value of C_i and a reduction in R_p (Fig. 3), but the maintenance of growth in e[CO_2] was not directly associated with the control of ABA levels in tissues. The ABA-deficient tomato mutant showed increases in biomass under e[CO_2] conditions possibly associated with increased in A and low R_P (Fig. 3A), while NCED mutants showed greater increments in biomass than MT plants under non-saline and saline conditions (Fig. 1). Thus, the increase in ABA levels in leaves of NCED plants may have resulted in the maintenance of water status due to greater control of stomatal closure, which allowed an efficient balance between carbon input and use for biomass gain under e[CO_2]. Stomatal closure induced by e[CO_2] may be associated with ABA-dependent signaling mechanisms that act in the guard cells (Chater et al., 2015; Hsu et al., 2018; Tian et al., 2015). In addition, e[CO_2] could induce biosynthesis of ABA in the tissues which would promote better regulation of plants to stress due to stomatal closure and improved water status (Fang et al., 2019; Yan et al., 2017). Indeed, plants overexpressing NCED grown under e[CO_2] showed significant increases in photosynthesis accompanied by a 50 % reduction in g_S ($P = 0{,}0009$) when compared to their control (-NaCl) under a[CO_2] (Fig. 3), suggesting that the e[CO_2] potentiated stomatal closure in these conditions. Moreover, g_S in NCED plants (+NaCl) was kept, in relative terms, equal to its control in e[CO_2]. No significant g_S reduction in MT plants (-NaCl) under e[CO_2] was observed and the g_S in MT under saline stress remained at the same levels as control plants (-NaCl), which was also observed in a previous study that showed that the stomatal response of plants to e[CO_2] may be more flexible in conditions of fluctuating temperature and irradiance conditions (Brito et al., 2020).

not plants showed high g_S values and stomatal aperture width in relation to the other genotypes (Fig. 3 and 4). Moreover, it is also noted that the Na^+ and K^+ concentration in tomato leaves was the same for both genotypes independent of ABA concentration in tissues under both a[CO_2] and e[CO_2] in saline conditions (Fig. 2), even with high transpiration rates due to high index and stomatal aperture in not mutants (Fig. 3, 4). Furthermore, leaves of not plants (-NaCl) under e[CO_2] showed an increase in the absorption of K^+ in relation to the other genotypes treated with NaCl (Fig. 2). ABA-deficient sitiens mutants of tomato under NaCl stress and a[CO_2] exhibit low Na/K ratio compared to WT (cv. Rheinlands Ruhm), since the K^+ transport from root to leaf was facilitated through the transpiration stream in the xylem (Poór et al., 2019). The low Na/K ratio in sitiens mutants suggests that the ionic stress was minimized in relation to WT (Póor et al., 2019). In addition, the modulation of stomatal closure induced by ABA may reduce the transpiration flow under e[CO_2] and regulate the absorption of Na^+ and K^+ in leaves, decreasing the Na/K ratio (Piñero et al., 2014; Yi et al., 2015; Yu et al., 2015). Here, we found that the stomatal regulation modulated by ABA and e[CO_2] does not directly control the selective transport of Na^+ and K^+ to the leaves through the transpiration stream under natural environmental conditions.

The reduction in g_S induced by saline stress observed here mainly in not plants suggests that other factors than ABA can induce stomatal closure in not mutants under salinity. The not mutants showed drastic reductions in the RWC compared to MT and NCED plants treated with NaCl grown under a[CO_2] or e[CO_2] conditions in the period from 10 am to midday. Interestingly, these plants were able to recover their water status at times with less transpiration demand, as in the pre-dawn (Fig. 4). ABA plays an essential role in the control of stomatal closure under saline stress (Osakabe et al., 2014). Plants with inefficient stomatal regulation, as in ABA-deficient mutants, tend to reduce their RWC throughout the day (Mulholland et al., 2003; Poór et al., 2019), and have lower cell pressure potential, due to intense dehydration caused by high g_S (Fang et al., 2019; Manzi et al., 2016). However, the imbalance of water status under water deficit events results in reduction in leaf turgor pressure, which causes stomatal closure (Mulholland et al., 2003; Wei et al., 2020). Accordingly, the improvement of leaf water status by the increase of RWC in not plants under e[CO_2] may explain the greater g_S under salt stress (Fig. 3C, 4B), which indicates that stomatal control in plants deficient in ABA under e[CO_2] was the result of improved leaf water status under saline stress conditions.

Tomato mutants with altered ABA levels showed changes in the availability of carbohydrates in leaves and roots coupled with changes in biomass gain, under control and saline conditions, independently of [CO_2] (Fig. 1 and 5). Under both [CO_2], ABA-deficient (not) plants grown under control and saline conditions showed a lower leaf concentration of soluble hexoses than MT and NCED plants (Fig. 5). Furthermore, not plants grown under both [CO_2] had high concentrations of sucrose compared to MT and NCED plants in non-saline and saline conditions (Fig. 5). The plant growth is the result of the balance between the sugars production from photosynthesis and their efficient relocation to the sink tissues, of which they will be respiratory substrates and signals of cell division and expansion processes (Balibrea et al., 2000; Osorio et al., 2014). The accumulation of sucrose and the low concentrations of hexoses in leaves of not plants under a[CO_2] and e[CO_2], at the end of the light period is a strong indication that low [ABA] may impair the sink activity, in parallel with the decrease in photosynthesis and the low growth, especially under saline stress (Fig. 1 and 5). However, NCED plants under control (-NaCl) and saline conditions had lower sucrose concentration at the end of the day and high growth and assimilation rates under both [CO_2]. Starch, one of the end products of photosynthesis, is synthesized and reserved in chloroplasts throughout the light period and its breakdown into free sugars during the night is necessary for regulation the growth of sink tissues (Stitt and Zeeman, 2012; Sulpice et al., 2009), and salinity may cause limitations in its metabolism, compromising the growth rate (Kempa et al., 2008; Poór et al., 2019). In turn, not plants treated with NaCl under e[CO_2] showed a slight reduction in sucrose concentration and increase in starch concentration and in R_N, these effects were accompanied by a low gain in leaf area and the total biomass (Fig. 1; 3; 5, S2). Higher growth of not plants treated with NaCl under e[CO_2] (Fig 1, S2) may provide evidence of reprogramming of carbohydrate metabolism connected to increase of sink activity and mitochondrial respiration. In plants under e[CO_2], the greater photoassimilates supply leads to the stimulation of respiratory metabolism for biomass gains (Gasparini et al., 2019; Leakey et al., 2009; Markelz et al., 2014), which contributes to mitigate the effects of salinity (Li et al., 2020). Furthermore, the different availability of hexoses in leaves, high in NCED and low in not plants under salinity in e[CO_2], may be attached to the accumulation of carbohydrates and its efficient allocation to roots, controlling the growth of plants with high ABA levels (Fig. 1, S2). Sucrose is the main sugar to be remobilized from the source to sink in most plants via phloem (Lawson and Matthews, 2020). In addition to the energy role, sucrose from mesophilic cells is discharged into the apoplast and can act as an osmoregulator, which leads to stomatal closure in high light demand (Daloso et al., 2016; Lawson et al., 2014). NCED plants showed low levels of sucrose at the end of the day as well as lower g_S, when compared to not plants under both [CO_2] and under non-saline and saline conditions (Fig. 3; 5), which indicate that ABA is necessary for stomatal closure mediated by the action of sucrose, irrespective of its osmotic effect. This is consistent with previous reports showing that ABA-deficient (sitiens) mutants are insensitive to increased sucrose concentration in the apoplast (Kelly et al., 2013). This insensitivity occurs because the sucrose act as a signaling molecule instead of an osmolyte during stomatal closure. When sucrose is degraded, hexokinases from guard cells sense the free hexoses through an ABA-dependent mechanism, triggering the stomatal closure (Kelly et al., 2013). Collectively, these data suggest that ABA modulates vegetative growth by differentiated allocation of carbohydrates and e[CO_2] can reduce the limitations of sink activity from the increased carbohydrates availability under saline stress.

The central metabolism, and consequently plant growth, is strongly regulated by the fine balance between the availability of carbon and nitrogen. Amino acids are important reserves of

C and N that regulate the survival of plants under stress conditions (Batista-Silva et al., 2019; Hildebrandt et al., 2015). Plants treated with NaCl under a[CO_2] showed a reduction in biomass and RGR accompanied by an increase in concentration of free amino acids, such as proline in leaves and roots (Fig. 1 and 6). Moreover, a visible decrease in the concentrations of amino acids was observed in all genotypes treated with NaCl under e[CO_2], coupled with improved growth ability (Fig. 1 and 6). Under saline stress, the carbon deprivation for metabolism in all genotypes under a[CO_2] may have resulted in plants with a high free amino acids concentration. Amino acids are important stocks of C and N that can be remobilized to intermediates of the TCA cycle and electron donors to sustain mitochondrial respiration under abiotic stress conditions (Bandehagh and Taylor, 2020; Woodrow et al., 2017). Proline is an important indicator of salt stress which it plays a role in reducing damage to cell membranes and act as a ROS scavenger (Almeida et al., 2014; Zhu et al., 2020). However, under e[CO_2] concentrations of proline were reduced in MT and not leaves, as well as in NCED roots under saline conditions (Fig. 6). Thus, it is reasonable to assume that plants under e[CO_2] have efficient reprogramming of energy metabolism to coordinate the use of amino acids and direct them to growth under conditions of salt stress.

5 Conclusions

Our results demonstrated that ABA biosynthesis plays a role in growth control and salt stress tolerance of tomato plants under fluctuating environment. However, the enhance of growth of NaCl-treated plants by e[CO_2] is not directly regulated by ABA. These findings provide some insight into how e[CO_2] can modulate photosynthetic rates and improve water status for growth of plants with altered ABA levels under salt stress conditions. Also, the metabolic rearrangement in the levels of sugars and amino acids, coupled with increased dark respiration are necessary to optimize growth of tomato plants under salinity under e[CO_2].

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Figures

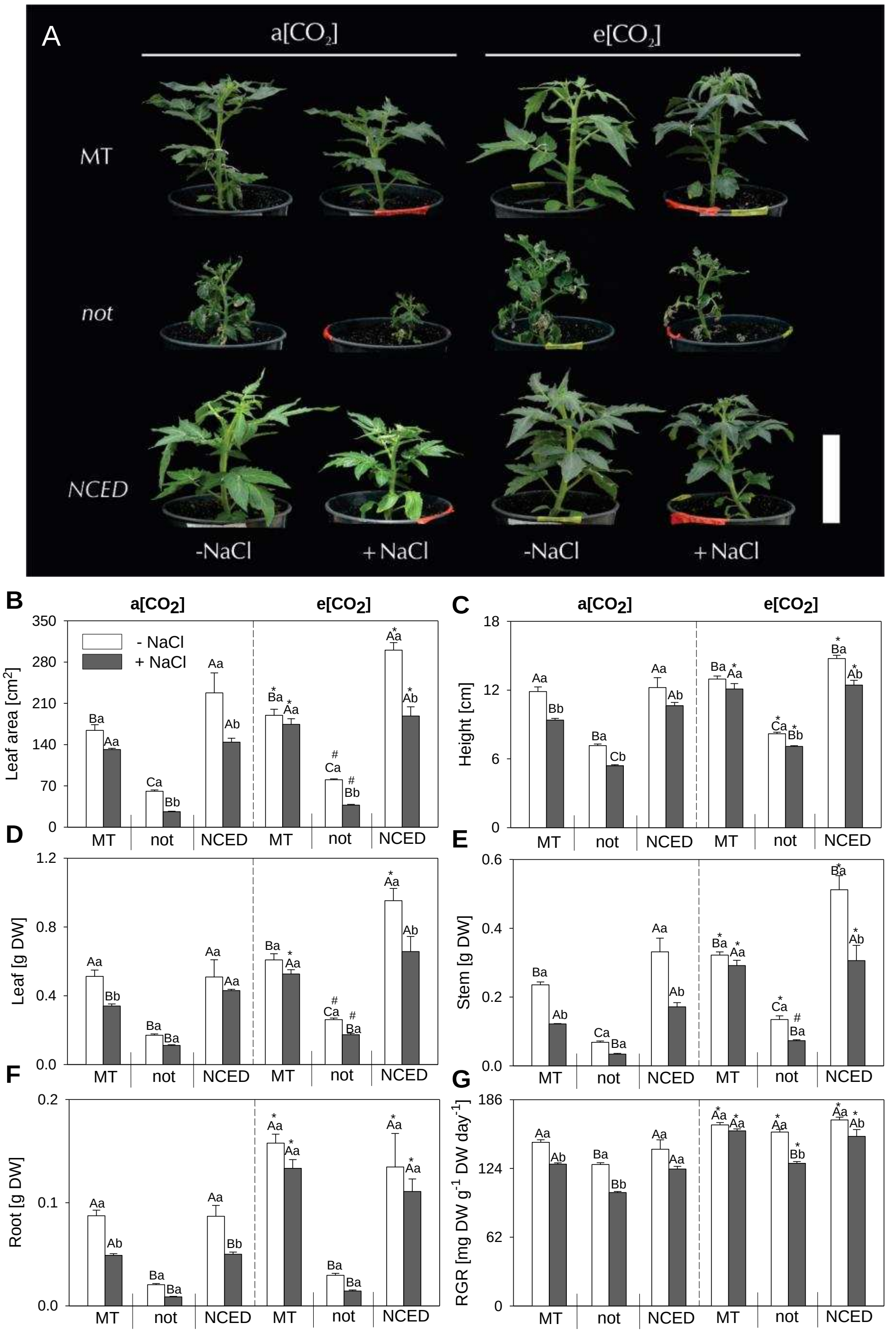

Fig. 1. Phenotypic changes in tomato plants cv. Micro-Tom (MT) and its respective notabilis (not) and LeNCED1 (NCED) mutants treated with NaCl grown under a[CO_2] and e[CO_2]. (A) Phenotypes of tomato plants after 21 days of 75 mM NaCl application grown under a[CO_2] and e[CO_2]; bar represents 10 cm scale. (B) Total leaf area. (C) Height of plants. (D) Weight of dry leaf. (E) Dry stem weight. (F) Weight of dry roots. (G) Relative growth rate (on a dry weight basis). Bars with different uppercase letters indicates significant differences between genotypes with the same treatment, lower case letters represent significant differences within genotypes with different treatment and asterisks (*) indicates differences between conditions under a[CO_2] and e[CO_2] by Tukey's test at 5% probability. The symbol # indicates significant difference within one genotype by simple ANOVA, not observed by triple factor analysis, by the Tukey test at 5% probability. Data are means and standard error of three separate experiments, and for each experiment 10 plants were harvested from each treatment.

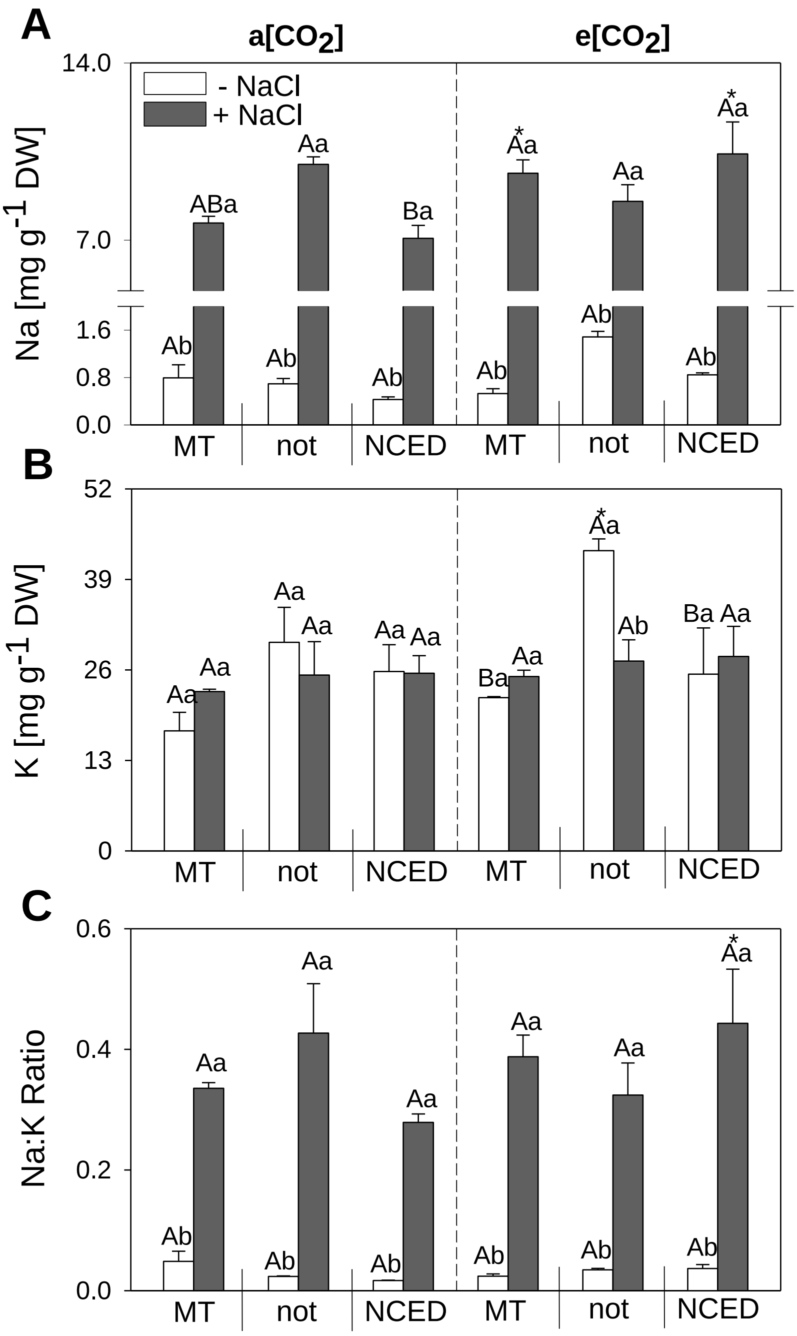

Fig. 2. Na^+ and K^+ concentration and Na^+:K^+ ratio in leaves of tomato cv. Micro-Tom (MT), notabilis (not) and LeNCED1 (NCED) treated with NaCl grown under a[CO_2] and e[CO_2]. (A) Na^+ in leaves. (B) K^+ in leaves. (C) Na^+:K^+ ratio in leaves. Bars with different uppercase letters indicates significant differences between genotypes with the same treatment, lower case letters represent significant differences within genotypes with different treatment and asterisks (*) indicates differences between conditions under a[CO_2] and e[CO_2] by Tukey's test at 5% probability. Data are means and standard error of three separate experiments, and for each experiment 6 plants were harvested from each treatment.

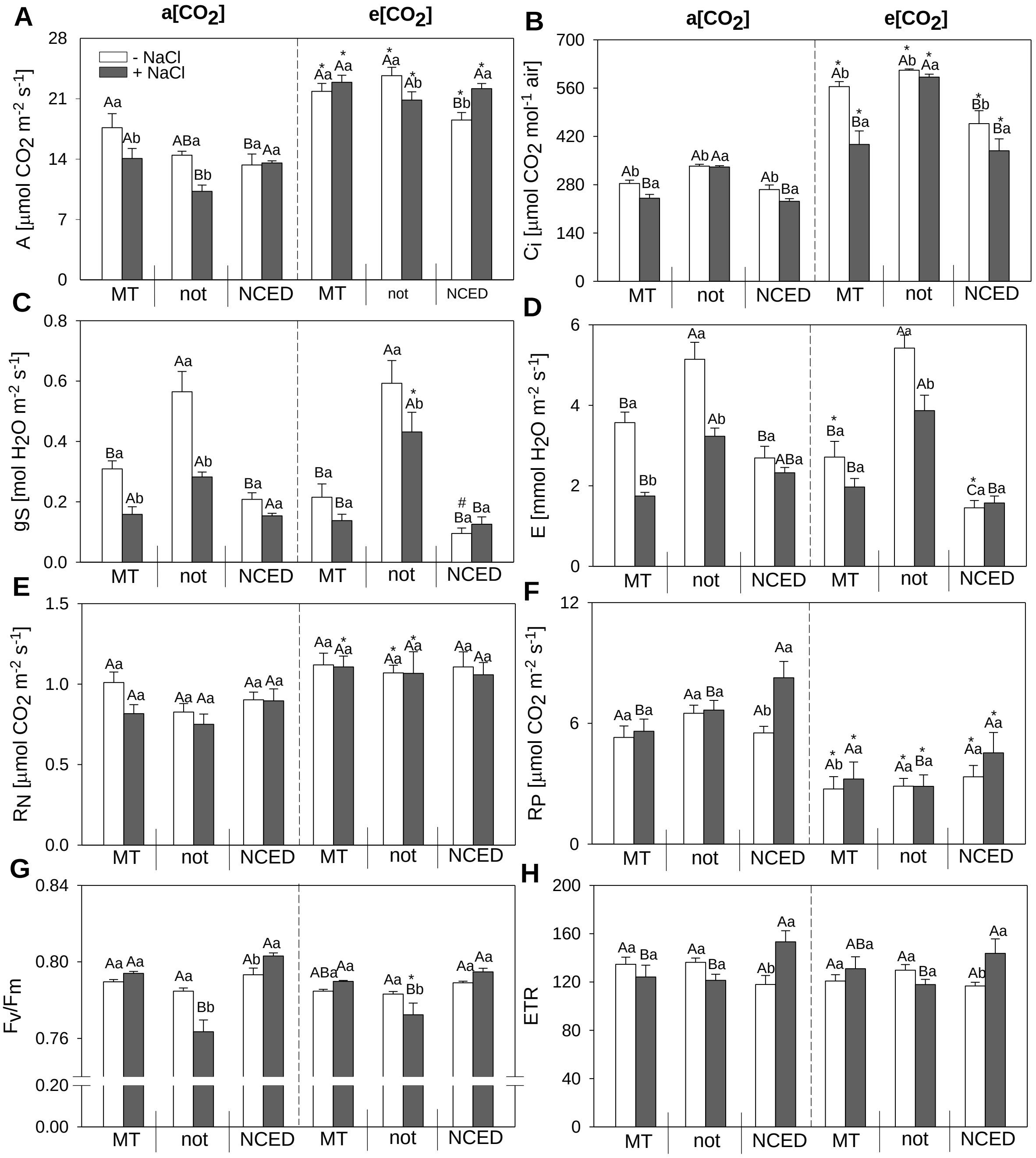

Fig. 3. Physiological parameters in leaves of tomato cv. Micro-Tom (MT), notabilis (not) and LeNCED1 (NCED) treated with NaCl grown under a[CO_2] and e[CO_2]. (A) Net CO_2 assimilation rate. (B) Intercellular CO_2 concentration. (C) Stomatal conductance. (D) Transpiration rate. (E) Dark respiration. (F) Photorespiration rate. (G) Electron transport rate. (H) Maximum photochemical efficiency of photosystem II. Bars with different uppercase letters indicates significant differences between genotypes with the same treatment, lower case letters represent significant differences within genotypes with different treatment and asterisks (*) indicates differences between conditions under a[CO_2] and e[CO_2] by Tukey's test at 5% probability. The symbol # indicates significant difference within one genotype by simple ANOVA, not observed by triple factor analysis, by the Tukey test at 5% probability. Data are means and standard error of three separate experiments, and for each experiment 6 plants were harvested from each treatment.

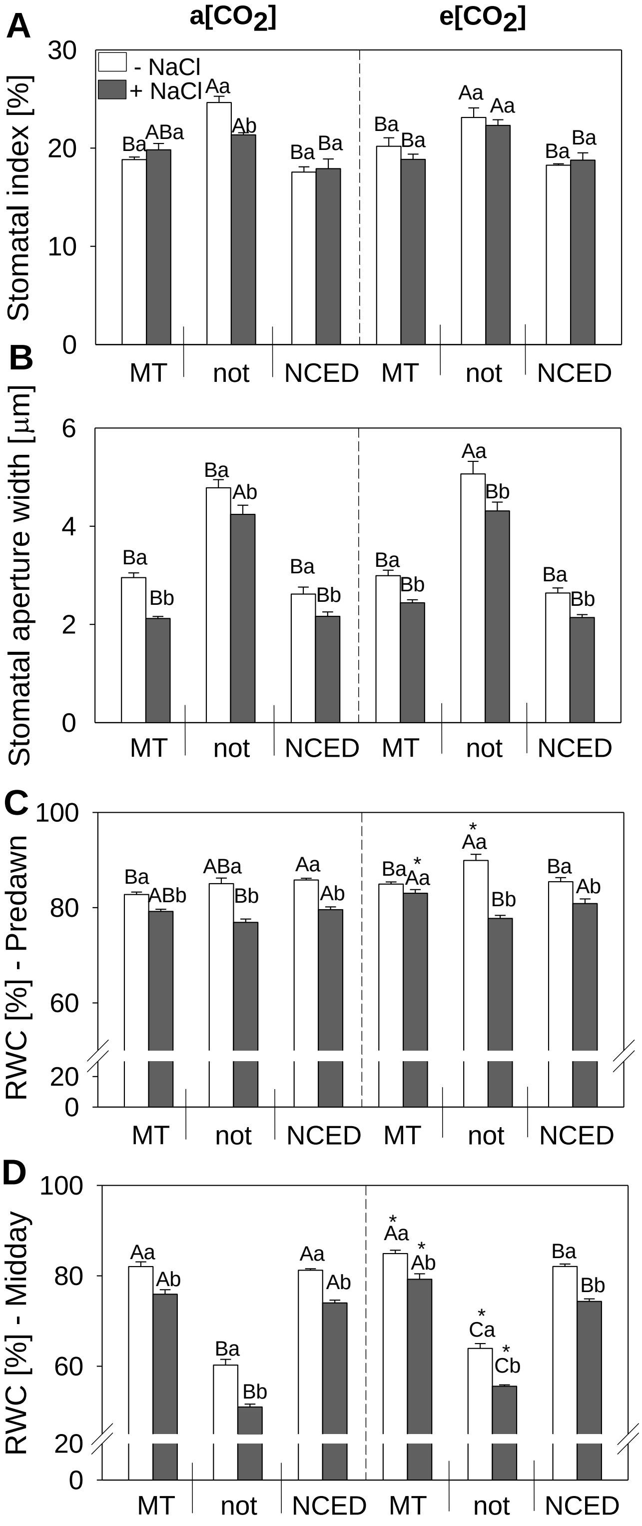

Fig. 4. Stomatal variations and water status of tomato plants cv. Micro-Tom (MT), notabilis (not) and LeNCED1 (NCED) treated with NaCl grown under a[CO_2] and e[CO_2]. (A) Stomatal index. (B) Stomatal aperture width. (C) Relative water content at pre-dawn. (D) Relative water content at midday. Bars with different uppercase letters indicates significant differences between genotypes with the same treatment, lower case letters represent significant differences within genotypes with different treatment and asterisks (*) indicates differences between conditions under a[CO_2] and e[CO_2] by Tukey's test at 5% probability. Data are means and standard error of three separate experiments with 6 replicates each.

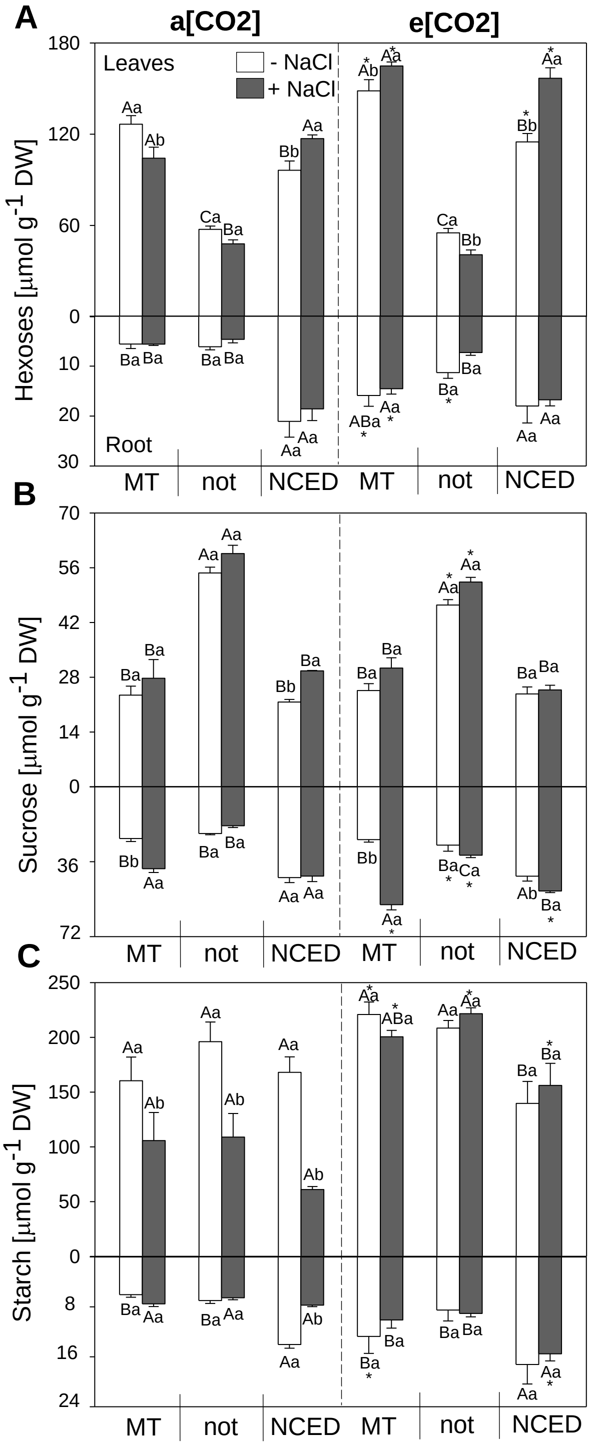

Fig. 5. Carbohydrate levels in leaves and roots of tomato plants cv. Micro-Tom (MT), notabilis (not) and LeNCED1 (NCED) treated with NaCl grown under a[CO_2] and e[CO_2]. (A) Hexoses (Glucose + Fructose). (B) Sucrose. (C) Starch. Bars with different uppercase letters indicates significant differences between genotypes with the same treatment, lower case letters represent significant differences within genotypes with different treatment and asterisks (*) indicates differences between conditions under a[CO_2] and e[CO_2] by Tukey's test at 5% probability. Data are means and standard error of three separate experiments with 6 replicates each.

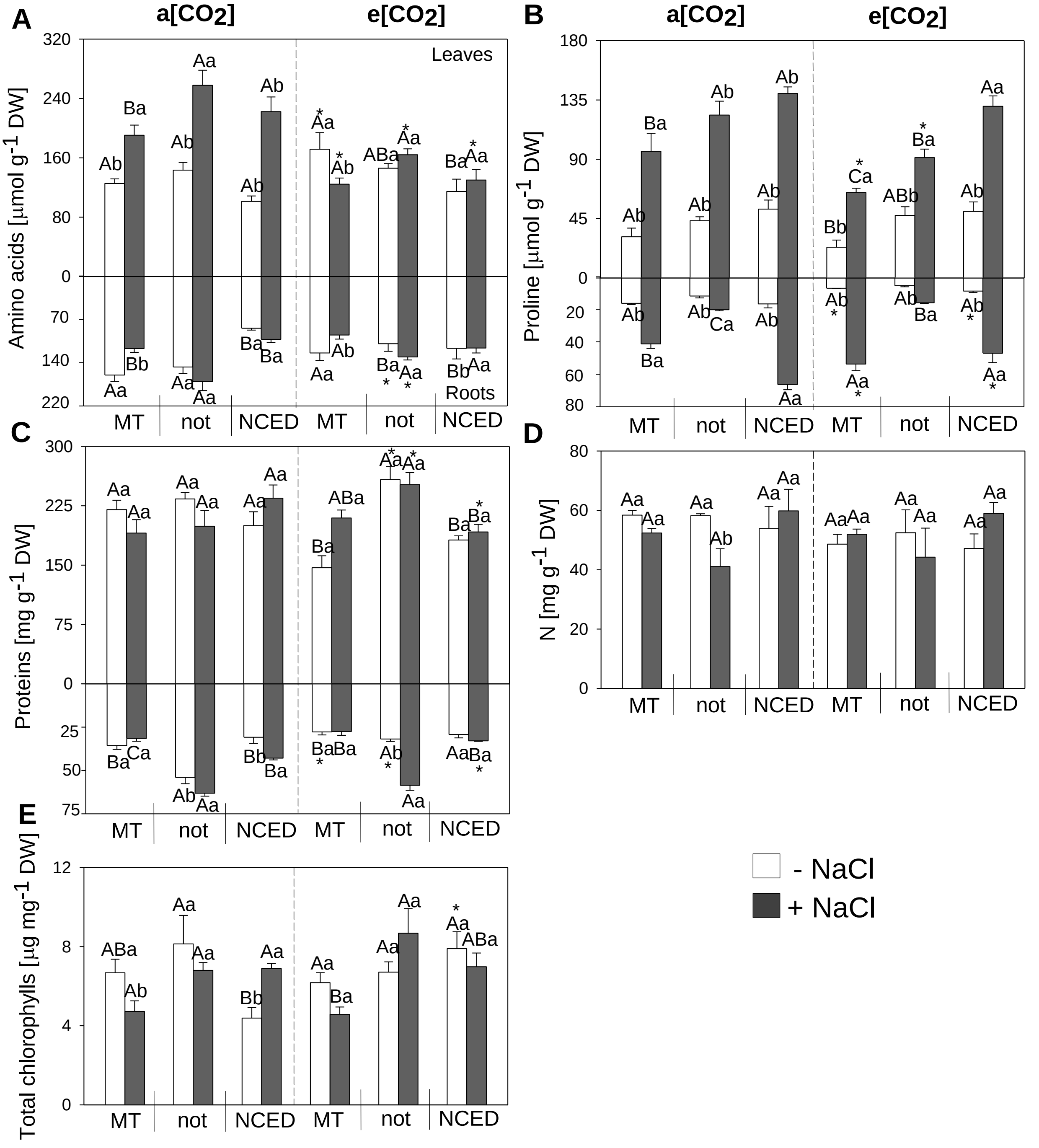

Fig. 6. Changes in the levels of nitrogen metabolites in leaves and roots of tomato plants cv. Micro-Tom (MT), notabilis (not) and LeNCED1 (NCED) treated with NaCl grown under a[CO_2] and e[CO_2]. (A) Total amino acids. (B) Proline. (C) Total soluble proteins. (D) Total nitrogen in leaves. (E) Total chlorophyll in leaves. Bars with different uppercase letters indicates significant differences between genotypes with the same treatment, lower case letters represent significant differences within genotypes with different treatment and asterisks (*) indicates differences between conditions under a[CO_2] and e[CO_2] by Tukey's test at 5% probability. Data are means and standard error of three separate experiments with 6 replicates each.

Table 1. Three-Way ANOVA (P-values) of significances and interactions between genotype (MT, not and NCED), treatment (-NaCl and + NaCl) and ambient (a[CO_2] and e[CO_2]). Data from replicates are analyzed from three separated experiments.

	Source of Variation (P value)						
	Genotype	Ambient	Treatment	Gen x Amb	Gen x Treat	Treat x Amb	Gen x Amb x Treat
Leaf area	**0.0000**	**0.0000**	**0.0000**	0.0574	**0.0002**	0.6569	0.4476
Height	**0.0000**	**0.0000**	**0.0000**	0.2863	0.5988	0.2199	0.0773
Dry Mass - Leaf	**0.0000**	**0.0000**	**0.0000**	**0.0005**	0.2354	0.3425	0.0716
Dry Mass - Stem	**0.0000**	**0.0000**	**0.0000**	**0.0039**	**0.0001**	0.8956	0.0903
Dry Mass - Root	**0.0000**	**0.0000**	**0.0002**	**0.0001**	0.4531	0.5469	0.826
RGR	**0.0000**	**0.0000**	**0.0000**	0.0515	**0.0155**	0.2393	0.2169
N	0.3125	0.2932	0.5954	0.8819	**0.0339**	0.2169	0.9688
Na^+	0.3217	**0.0013**	**0.0000**	**0.0013**	0.5232	**0.0192**	**0.0003**
K^+	**0.0033**	0.0622	0.4428	0.4313	**0.0195**	0.3825	0.3931
Na:K	0.4387	**0.0402**	**0.0000**	0.8701	0.794	**0.0248**	0.9885
A	**0.0036**	**0.0000**	0.0923	**0.0286**	**0.0007**	**0.0061**	0.479
g_S	**0.0000**	0.5816	**0.0000**	**0.0126**	**0.0025**	0.0518	0.9114
Ci	**0.0000**	**0.0000**	**0.0000**	**0.0037**	**0.0073**	**0.0098**	0.1584
Fv/Fm	**0.0000**	0.1662	0.4928	**0.0443**	**0.0000**	0.4911	0.2128
R_D	0.2558	**0.0000**	0.1913	0.6181	0.7442	0.4152	0.5739
E	**0.0000**	0.0549	**0.0000**	**0.001**	**0.0008**	**0.0327**	0.6533
Ci/Ca	**0.0000**	0.1114	**0.0000**	0.0726	**0.0192**	0.7231	0.1165
ETR	0.4007	0.2715	0.1644	0.9806	**0.0002**	0.5395	0.364
R_P	**0.0301**	**0.0000**	**0.0273**	0.3747	0.0804	0.4809	0.5843
Stomatal index	**0.0000**	0.5435	0.119	0.5202	**0.0245**	0.8882	**0.041**
Stomatal aperture	**0.0000**	0.1383	**0.0000**	0.5712	0.5107	0.9668	0.4428
RWC pre-dawn	0.6058	**0.0000**	**0.0000**	**0.0434**	**0.0000**	0.7837	**0.0157**
RWC midday	**0.0000**	**0.0000**	**0.0000**	**0.0118**	0.0555	0.7771	0.8257
Hexoses - Leaf	**0.0000**	**0.0000**	0.0633	**0.0000**	**0.0000**	**0.0025**	**0.012**
Hexoses - Root	**0.0000**	**0.0007**	0.0903	**0.0001**	0.7055	0.6666	0.7421
Sucrose - Leaf	**0.0000**	0.0583	**0.0001**	**0.0069**	0.9705	0.545	0.3011
Sucrose - Root	**0.0000**	**0.0000**	**0.0000**	**0.0349**	**0.0000**	**0.0000**	0.2206
Starch - Leaf	**0.0001**	**0.0000**	**0.0001**	0.1565	0.9284	**0.0000**	0.1406
Starch - Root	**0.0000**	**0.0000**	0.0876	0.2164	0.0791	0.7777	0.1627
Amino acids - Leaf	**0.0006**	**0.0002**	**0.0000**	0.1149	**0.0381**	**0.0000**	0.4892
Amino acids - Root	**0.0000**	**0.0276**	0.6446	**0.011**	**0.0000**	0.7645	0.845
Protein - Leaf	**0.0002**	0.4458	0.4603	**0.0015**	0.0761	0.0572	**0.0191**
Protein - Root	**0.0000**	**0.0000**	**0.0000**	**0.0315**	**0.0000**	0.0914	**0.0008**
Proline - Leaf	**0.0000**	**0.0036**	**0.0000**	0.4122	**0.0162**	**0.0116**	0.4168
Proline - Root	**0.0000**	**0.0003**	**0.0000**	**0.0005**	**0.0000**	0.1649	**0.0001**
Chlorophylls	**0.002**	0.1221	0.6022	0.1173	**0.0492**	0.823	**0.0085**

Supplementary material

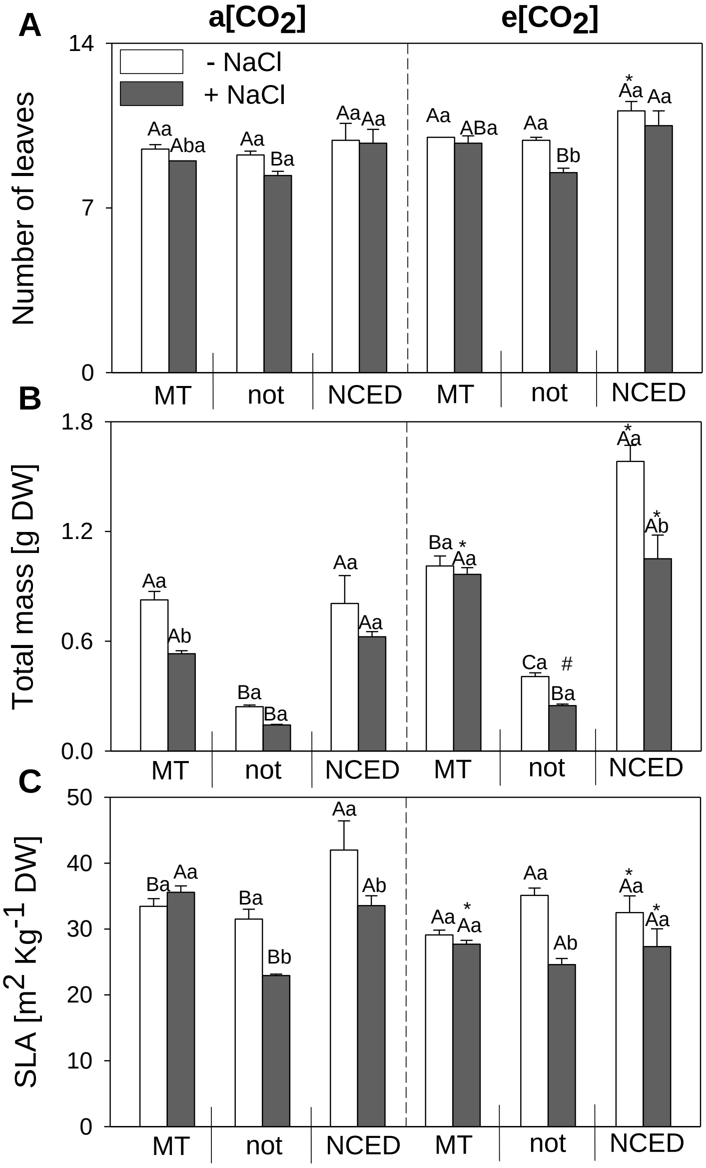

Fig. S1. Phenotypic changes in tomato plants cv. Micro-Tom (MT) and its respective notabilis (not) and LeNCED1 (NCED) mutants treated with NaCl grown under a[CO_2] and e[CO_2]. (A) Number of leaves. (B) Total dry mass. (C) Specific leaf area. Bars with different uppercase letters indicates significant differences between genotypes with the same treatment, lower case letters represent significant differences within genotypes with different treatment and asterisks (*) indicates differences between conditions under a[CO_2] and e[CO_2] by Tukey's test at 5% probability. The symbol # indicates significant difference within one genotype by simple ANOVA, not observed by triple factor analysis, by the Tukey test at 5% probability. Data are means and standard error of three separate experiments, and for each experiment 10 plants were harvested from each treatment.

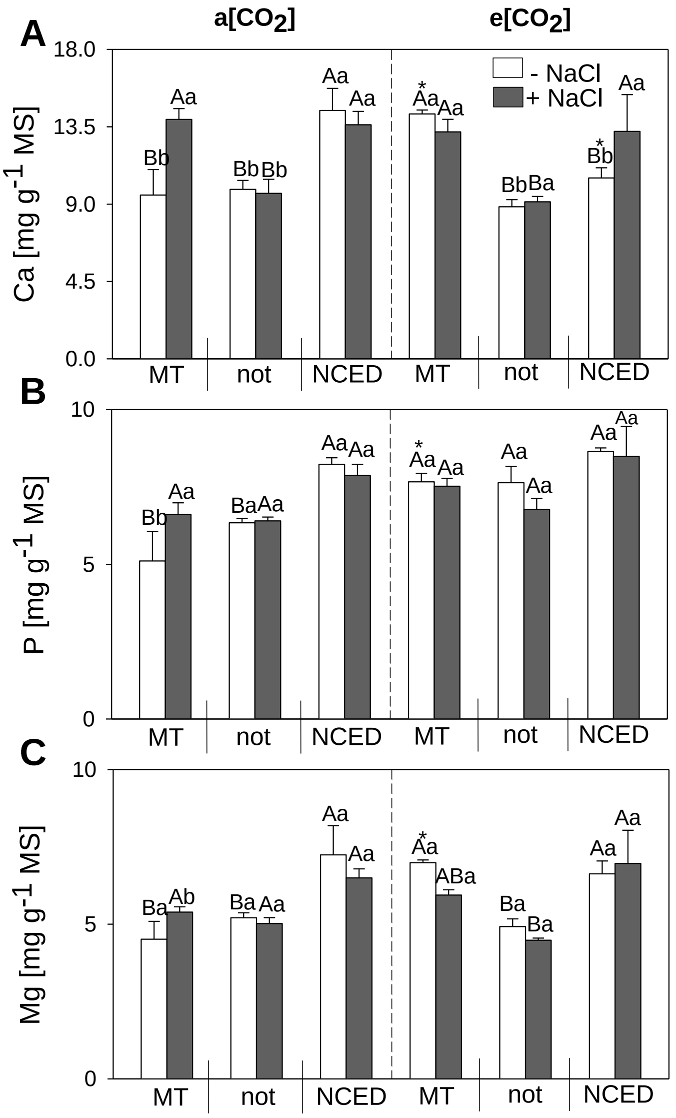

Fig. S2. Leaf mineral composition of tomato cv. Micro-Tom (MT), notabilis (not) and LeNCED1 (NCED) treated with NaCl grown under a[CO_2] and e[CO_2]. (A) Ca^{2+} in leaves. (B) P^{3+} in leaves. (C) Mg^{2+} in leaves. Bars with different uppercase letters indicates significant differences between genotypes with the same treatment, lower case letters represent significant differences within genotypes with different treatment and asterisks (*) indicates differences between conditions under a[CO_2] and e[CO_2] by Tukey's test at 5% probability. Data are means and standard error of three separate experiments, and for each experiment 6 plants were harvested from each treatment.

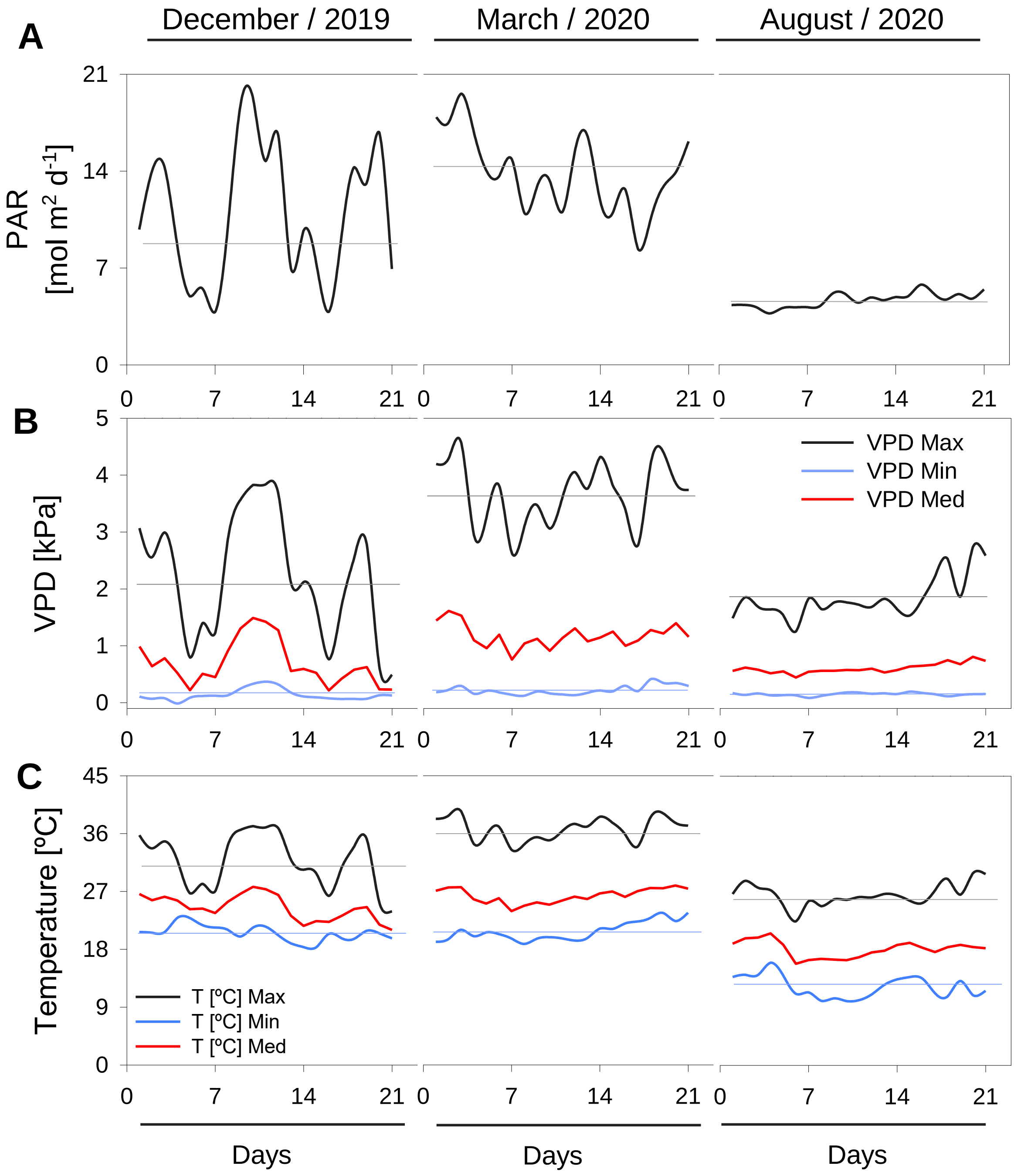

Fig. S3. Fluctuation of daily global irradiance, air temperature and vapour pressure deficit for 21 days of each experiment. (A) Daily photosynthetically active radiation inside the two open-top chambers supplemented with ambient (a[CO_2]) and elevated (e[CO_2]) CO_2 concentration. (B) Vapour pressure deficit inside the two open-top chambers supplemented with ambient (a[CO_2]) and elevated (e[CO_2]) CO_2 concentration. (C) Air temperature inside the two open-top chambers supplemented with ambient (a[CO_2]) and elevated (e[CO_2]) CO_2 concentration.

Table S1. Three-Way ANOVA (P-values) of significances and interactions between genotype (MT, not and NCED), treatment (-NaCl and + NaCl) and ambient (a[CO_2] and e[CO_2]). Data from replicates are analyzed from three separated experiments.

	S.V. (P Value)						
	Genotype	Ambient	Treatment	Gen x Amb	Gen x Treat	Treat x Amb	Gen x Amb x Treat
Total dry Mass	**0**	**0**	**0**	**0**	0.0465	0.4987	**0.0105**
SLA	0.0007	0.0009	0	0.0003	0.0012	0.7421	0.4156
Number of Leaves	**0**	**0.0026**	**0.0046**	0.4921	0.2636	0.5621	0.7137
Ca	**0**	0.5996	0.1331	**0.02**	0.5067	0.6865	**0.0116**
P	**0.0001**	**0.0011**	0.9794	0.1949	0.2452	0.1675	0.4036
Mg	**0**	0.236	0.4786	**0.0221**	0.9462	0.5204	0.1118

GENERAL CONCLUSION

The present study demonstrated that the negative effects of salinity on tomato plants were minimized by e[CO_2] under conditions of environmental fluctuations of irradiance, humidity and temperature. e[CO_2] enhances photosynthesis associated with reductions of ABA and ACC, which allowed plants under saline stress to maintain a high stomatal conductance. In addition, growth increments in plants under saline stress is determined by increases in respiratory activity under e[CO_2]. In relation to the ABA content in tissues, e[CO_2] allows an increase in photosynthesis and gains in biomass of plants deficient in the biosynthesis of ABA (not) and plants with higher biosynthesis of ABA (NCED) under salinity conditions. Saline stress tolerance of not and NCED plants under e[CO_2] was mediated by changes in the primary metabolism associated with the induction of sink capacity. This suggests that e[CO_2] minimizes the effects of salt stress by ABA-independent mechanisms.