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Mild salt stress improves strawberry fruit quality

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ABSTRACT

Strawberry is one of the most popular fruits because of its shape, color and taste, and the presence of antioxidant compounds. Because severe abiotic stresses result in detrimental consequences to plant growth, the effect of cultivating under mild stress conditions has rarely been investigated. Therefore, we evaluated the effect of mild salt stress on yield and quality of strawberry fruit. Mild salt stress did not affect yield. The lower level of mild salt stress evaluated showed increased vegetative growth (24%), higher photosynthetic effectiveness, and increased activity of phenoloxidase (22%) and polyphenoloxidase (33%), as well as the accumulation of sucrose (5%) and anthocyanins (60%) in the fruit, compared to non-stressed plants. The higher level of mild salt stress increased root growth (30%), the activity of phenylalanine ammonia lyase (68%), and the accumulation of total phenolic compounds (14%), and total antioxidant activity (13%) in the fruit, compared to non-stressed plants. The only phenolic compound improved in these treatments was (+)- catechins. Both levels of salt stress affected the expression of genes involved in the phenylpropanoid and flavonoid pathways, cell wall disassembly and abscisic acid-related genes. Therefore, mild salt stress improves strawberry fruit quality.

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1. Introduction

To meet increasing food demands under the anticipated scenario of climatic change, it will be necessary to expand our agricultural systems to drier and more saline lands. Intensive production systems such as soilless culture, in which saline solutions supply nutrients to plants, have recently increased in use to address intensified food market demand. Moreover, due to the reduction in water resources available for crop production, there will be an increase in the use of poor quality irrigation water in the future (Huang et al., 2012; Krasensky & Jonak, 2012). Therefore, it is of great importance to evaluate the effect of salt stress on crop development.

Although severe abiotic stresses such as salt stress result in detrimental consequences to plant growth, mild stress conditions may be deliberately applied to improve the content of antioxidant compounds in the edible part of the plant, and stimulate plant adaptation to stress-prone environments (Kim, Fonseca, Choi, Kubota, & Kwon, 2008; Cogo et al., 2011; reviewed by; Ripoll et al., 2014). The consumption of fruit and vegetables containing antioxidant compounds is positively associated with the prevention of several chronic diseases and the improvement of general health; therefore, efforts to increase the nutritional and functional quality of foods during plant cultivation, an approach known as biofortification, are of great interest (Messias, Galli, Silva, Schirmer, & Rombaldi, 2013; Zhu et al., 2013).

The strawberry (*Fragaria* × *ananassa* Duch.) is one of the most popular fruits because of its taste and well-recognized health-promoting properties due to the presence of potential functional compounds such as phenolic compounds (Giamperi et al., 2012). Its demand and availability in the market has widely increased, making this fruit a target of biofortification efforts. Strawberry plants are sensitive to salt stress, but are cultivated using fertilizers and water of inadequate quality for irrigation, resulting in a gradual buildup of salt in the soil (Jamalian, Gholami, & Esna-Ashari, 2013). Despite this fact, the effect of cultivating strawberry plants under mild salt stress conditions has rarely been investigated, especially the effects on fruit quality. Moreover, the molecular mechanisms underlying the effects of mild stress remain to be elucidated. Therefore, in the present study, we evaluated the effects of different levels of mild salt stress on several biochemical, physiological and molecular aspects of plant growth and the yield and quality of

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strawberry fruit, as well as the mechanisms underlying these effects.

2. Materials and methods

2.1. Experimental design and treatments

The experiment was conducted in a greenhouse at the Brazilian Agricultural Research Corporation (Embrapa Temperate Agriculture/Pelotas/RS/Brazil). Strawberry seedlings of the Camarosa cultivar were transplanted and grown in 9 L pots containing a mixture of soil (Ultisoil, 6.6 kg/pot) and vermiculite (2.2 kg/pot). The fertilizer was composed of urea, triple superphosphate and potassium chloride as sources of 267 kg/ha N, 619 kg/ha P_2O_5 and 333 kg/ha K_2O, respectively. Irrigation was performed by a drip irrigation system through daily dripping. The relative soil moisture was always maintained between 16 and 19% (soil saturation) with no water leaching.

The experimental design was completely randomized with three treatments and four replicates per treatment, as follows: C (control); L1 (stress level 1 – salt solution of 40 mmol/L NaCl in distilled water); L2 (stress level 2 - salt solution of 80 mmol/L NaCl in distilled water). For the salt stress treatments (L1 and L2), 50 mL of salt solution was applied once a week from the beginning of the flowering stage (105 days after transplanting - DAT) to the end of the crop cycle (190 DAT). The same volume of distilled water was applied in the C treatment.

Mature fruits (fully red, according to Jia et al. (2011)) were sampled at the end of the experiment, frozen in liquid nitrogen, and stored at −80 °C until analyzed. The experimental timeline is shown in Fig. 1.

2.2. Soil electrical conductivity

Soil electrical conductivity was determined using a conductivimeter (Tecnal, TEC-4MPP model). The samples were diluted in distilled water (1:5 v/v) prior the quantification. These measurements were performed after two (measurement M1), seven (measurement M2) and ten (measurement M3) applications of the treatments (Fig. 1), and are presented in microsiemens per centimeter (μS/cm).

2.3. Photosynthetic parameters

The CO_2 assimilation rate of the plants was monitored with a portable gas exchange fluorescence system infrared gas analyzer (IRGA) (Heinz Walz GmbH, GFS 3000 model), using 500 ppm of CO_2 and 800 ppm of light as parameters. Five measurements (M1 to M5) (Fig. 1) were performed during the crop cycle, at the same time of the day (from 11:00–14:00), and using new, fully developed leaves from three of the six plants in each replicate. The results are presented as mmol/m^2/s.

2.4. Yield of fruit, fresh biomass and root biomass

For the determination of crop yield, fruits in the commercial stage of ripening (full red, according to Jia et al., 2011) were sampled and weighed throughout thecrop cycle. Fresh plant biomass was determined at the end of the cycle by weighing the aboveground portion of the plant. The underground portion of the plant was also weighed to quantify root biomass. The results are expressed in grams per plant (g/plant).

2.5. Content of sodium (Na) and chloride (Cl) in strawberry leaves

The content of Na and Cl was determined in dried leaves, according to the method described by Tedesco, Gianello, Bissani, Bohnen, and Volkweiss (1995). These measurements were performed in four biological replicates and three analytical replicates. The results are expressed as grams per kilogram of leaves (g/kg).

2.6. Quality traits of strawberry fruit

The method described by Nelson (1944) was used to quantify and reducing sugars in lyophilized strawberry fruit. Samples were subjected to an acid hydrolysis to determine the total content of sugars. The difference between the total content of sugars and the content of reducing sugars was considered to correspond to the content of sucrose. The results are expressed as g/kg. Total phenolic content was quantified following the method from Swain and Hillis (1959). The data are presented as grams of gallic acid equivalents per kilogram of fruit (g GAE/kg fruit). The total anthocyanins content in the strawberry fruit was determined according to Zhang, Pang, Yang, and Jiang (2004). The data are presented as grams of pelargonidin equivalents per kilogram of fruit (g PE/kg fruit). The methodology described by Brand-Williams, Cuvelier, and Berset (1995) and Arnao, Canoa, and Acosta (2001) was used to determine the total antioxidant activity. The data are presented as mmol/L of Trolox equivalents per grams of fruit (mmol/L TE g fruit^{-1}).

Individual phenolic compounds were quantified from 0.5 g of the lyophilized sample suspended in 30 mL of methanol. Next,

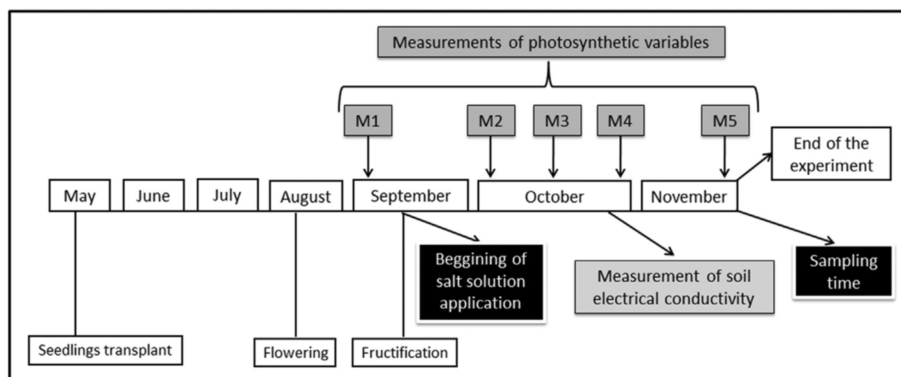

Fig. 1. Timeline of the experiment with strawberry plants. Strawberry plants (Camarosa cv.) were cultivated under mild salt stress. The measurement of soil electrical conductivity and photosynthetic variables (M1 to M5) performed during the experiment are represented by light and dark gray squares, respectively. The beginning of the salt stress application and the sampling dates of fruit and leaves used in biochemical and molecular measurements are represented by black squares.

4.9 mL 0.1 mol/L hydrochloric acid was added to the extract and homogenized for 24 h at 35 °C, in the dark. The extract was filtered and the supernatant was concentrated in rotary evaporator at 40 °C for 30 min. The residue was concentrated and resuspended in methanol to obtain a final volume of 5 mL, which was then centrifuged at 5500 × g for 10 min. The supernatant (30 μL) was injected into the chromatograph. We used a liquid chromatography system (HPLC - Shimadzu LC-10AT) with an auto sampler, UV–visible detector at 280 nm, RP-18 CLC- ODS reverse phase column (5 μm, 4.6 mm × 150 mm) with an octadecylsilane stationary phase and a CLC- GODS guard column. The mobile phase gradient elution consisted of an aqueous solution containing 990 mL/L acetic acid and 10 mL/L methanol, with a flow rate of 0.8 mL/min and a total run time of 45 min. Standards for the quantification of gallic acid, (+) - catechin, *p*-hydroxycinnamic acid, caffeic acid, *p*-coumaric acid and ellagic acid were obtained from Sigma Aldrich. The results are expressed in g/kg of fruit in DW.

All analyses were performed in the four biological replicates and three analytical replicates.

2.7. Phenylalanine ammonia lyase (PAL), peroxidase (POD) and polyphenoloxidase (PPO) activity in strawberry fruit

The determination of PAL activity was performed according to the method described by Campos et al. (2003), which involved incubating the samples in borate buffer pH 8.5 and 60 μmol/L phenylalanine at 45 °C. The activity of peroxidase (PO, EC 1.11.1) and polyphenoloxidase (PPO, EC 1.14.18.1) were determined according to the method described in Campos et al. (2004). For both analyses, samples were extracted with 0.05 mol/L phosphate buffer (pH 7.0) and polyvinylpyrrolidone. For PO determination, 1.5 mL of the extract was incubated at 30 °C with 2.5 mL citrate-phosphate buffer (0.2 mol/L sodium phosphate and 0.1 mol/L citric acid, pH 5.0), and 0.25 mL 0.05 mol/L guaiacol. For PPO analysis, 1 mL of the extract was incubated at 30 °C with 3.6 mL 0.05 mol/L phosphate buffer (pH 6.0) and 0.1 mL 0.1 mol/L catechol. The analyses were performed in four biological replicates and three analytical replicates. The results are expressed as microkatals of transcinnamic acid per kilogram of fruit (μKat/kg).

2.8. Gene expression analysis in strawberry fruit

The isolation of total RNA, cDNA synthesis and amplification by RT-qPCR were performed as described in Galli et al. (2015). Specific primers were designed to amplify the desired sequences, as shown in Table S1. The reference genes *PIRUV_DESCARB* (pyruvate decarboxylase), and *HISTH4* (histone H4) were used to normalize the transcript levels. These genes were selected according to Galli et al. (2015). The relative expression data were calculated according to the $2^{-\Delta\Delta Cq}$ method and are presented as relative expression. The analysis was performed for four biological replicates and three analytical replicates.

2.9. Statistical analyses

Statistical analyses were performed using SAS System for Windows version 9.1.3. Data were subjected to variance analysis ($P \leq 0.05$). To assess statistical significance, we compared the treatments using Tukey's test ($P \leq 0.05$). Pearson's correlation (r) analysis was performed to correlate variables ($P \leq 0.05$). The data were recorded as the mean ± s.d.

3. Results and discussion

3.1. Mild salt stress did not affect the yield of strawberry plants

In this study, two increasing doses of mild salt stress (L1 and L2) were applied to strawberry plants. The presence of increased contents of Na and Cl in the strawberry leaves and the increased electrical conductivity of the soil compared to the control (Table 1) confirmed the successful implementation of the treatments.

However, the yield of strawberry fruit was not statistically ($P < 0.05$) affected by the two levels of stress (Table 1), suggesting that these salt levels are of mild intensity and that Camarosa cv. possesses the ability to tolerate the levels of salinity evaluated through osmotic regulation; this tolerance behavior of Camarosa cv. was previously described by Kaya, Kirnak, Higgs, and Saltali (2002). Moreover, the implementation of salt stress during the flowering to fruiting stages of plant development (see Fig. 1) may also have contributed to the low level of damage observed in the plants because most of the deleterious effects of abiotic stresses are observed when stress is inflicted during the initial vegetative stages (Ripoll et al., 2014).

3.2. The lower level of mild salt stress positively affected photosynthesis and fresh biomass

The fresh biomass of plants in the L2 treatment group (21 ± 3 g/plant) was significantly lower than that of plants in the control group (27 ± 1.7 g/plant) (Table 1), confirming previous reports showing negative effects of salt stress on vegetative development (Turhan & Eris, 2009). However, the fresh biomass in the plants of L1 group (33 ± 1.7 g/plant) was statistically higher ($P < 0.05$) than that of L2 and C, suggesting that the mild stress from L1 resulted in beneficial effects for leaf production, a result that could be of interest in the production of leafy vegetables. Similar results were observed for CO_2 assimilation rates, in which L1 showed significantly higher values ($P < 0.05$) compared to the other treatments for the M2 and M3 measurements (Table 2), suggesting higher photosynthetic rates. Photosynthetic rates are usually reduced by salinity in most plant species (Nawaz et al., 2010); however, increased photosynthetic rates resulting from low levels of salt stress were also observed by Sun et al. (2011) in *Periploca sepium* plants.

The increased strawberry biomass observed under mild stress (L1) may be associated with increased photosynthetic rates and may be explained in terms of hormesis, which has been proposed as an adaptive response to low levels of stress or damage, resulting in improved fitness for some physiological systems (Wiegant, Poot, Boers-Trilles, & Schreij, 2013). New insights into the improvement of plant biomass under salt stress conditions are under investigation, including the role of components of the cellulose synthase complex (Endler et al., 2015).

3.3. Mild salt stress improves the content of antioxidant compounds and sucrose in the fruit

The accumulation of antioxidant compounds in fruits is considered of great importance, particularly because of the health benefits said to arise from their ingestion in the diet (Giamperi et al., 2012; Messias et al., 2013). In this context, mild salt stress seems to be an effective biofortification effort: the L1 group showed induced accumulation of anthocyanins ($P < 0.05$), and the total antioxidant activity was significantly ($P < 0.05$) increased in strawberry fruits from L2, which was associated with an increased content of total phenolic compounds (99% correlation) (Table 3).

The content and composition of individual phenolic acids

Table 1
Soil electrical conductivity, sodium content in leaves, chlorine content in leaves, fruit yield, fresh biomass and fresh root biomass of strawberry plants under mild salt stress.

Treatments	Soil electrical conductivity (μSm/cm)	Sodium content (g/kg)	Chloride content (g/kg)	Fruit yield (g/planta)	Fresh biomass (g/planta)	Fresh root biomass (g/planta)
C	416 ± 38c	0.184 ± 0.03c	3.7 ± 0.6c	197 ± 15 ns	26.6 ± 1.7b	7.8 ± 1.4b
L1	739 ± 64b	0.296 ± 0.01b	7.4 ± 0.6b	206 ± 17	33.1 ± 1.7a	8.4 ± 1.4ab
L2	1091 ± 95a	0.734 ± 0.03a	12.1 ± 1.0a	182 ± 16	21.0 ± 3.3c	10.2 ± 0.6a

Strawberry plants (Camarosa cv.) were subjected to the application of distilled water (C), 40 mmol/L NaCl (L1) or 80 mmol/L NaCl (L2). The soil was diluted 1:5 (mL:mL) in distilled water for determination of soil electrical conductivity, and the measurements were performed according to Fig. 1. The yield of strawberry fruit was obtained by weighing all fully red fruit during the experiment. The fresh biomass and fresh root biomass were measured at the end of the experiment. The means of four replicates ± SD are shown. Different letters indicate significant differences ($P < 0.05$, Tukey's test) between treatments within a single column. ns - not significant.

Table 2
CO_2 assimilation rate of strawberry plants under mild salt stress.

Treatments	Measurements of CO_2 assimilation rates (mmol/m_2/s)				
	M1	M2	M3	M4	M5
C	15.7 ± 1.4 ns	10.3 ± 1.0b	10.9 ± 0.6b	7.8 ± 0.5 ns	12.1 ± 1.6 ns
L1	16.8 ± 0.8	13.9 ± 1.3a	14.7 ± 0.8a	7.8 ± 0.9	10.0 ± 1.8
L2	16.1 ± 1.4	11.0 ± 0.5b	9.9 ± 0.8b	8.8 ± 2.0	11.9 ± 0.7

Strawberry plants (Camarosa cv.) were subjected to the application of distilled water (C), 40 mmol/L NaCl (L1) or 80 mmol/L NaCl (L2). The measurements (M1 to M5) were performed according to Fig. 1. The means of four replicates ± SD are shown. Different letters indicate significant differences ($P < 0.05$, Tukey's test) between treatments within a single column. ns - not significant.

Table 3
Quality traits and enzymatic activity of strawberry fruit cultivated under mild salt stress.

Treatments	Reducing sugars (g/kg)	Sucrose (g/kg)	Anthocyanins (g PE/kg)	Antioxidant Activity (mmol TE/kg)	Phenolic compounds (g GAE/kg)	PPO activity (μKat/kg)	PO activity (μKat/kg)	PAL activity (μKat/kg)
C	15.66 ± 0.3 ns	20.62 ± 0.17b	0.87 ± 0.05b	35.7 ± 3.0b	3.54 ± 0.05b	2.5 ± 0.3b	2.13 ± 0.01b	1.4 ± 0.2b
L1	15.81 ± 0.3	21.56 ± 0.60a	1.39 ± 0.11a	37.1 ± 3.7ab	3.64 ± 0.22b	3.4 ± 0.2a	2.59 ± 0.19a	1.8 ± 0.1b
L2	15.16 ± 0.2	20.12 ± 0.04b	1.07 ± 0.03b	40.2 ± 1.5a	4.03 ± 0.23a	2.9 ± 0.4ab	2.27 ± 0.05ab	2.4 ± 0.3a

Strawberry plants (Camarosa cv.) were subjected to the application of distilled water (C), 40 mmol/L NaCl (L1) or 80 mmol/L NaCl (L2). The means of four replicates ± SD are shown. All analysis were performed on dry weight. Different letters indicate significant differences ($P < 0.05$, Tukey's test) between treatments within a single column. PE – pelargonidine equivalent; TE – trolox equivalent; GAE – gallic acid equivalent; PPO – polyphenoloxidase; PO – phenoloxidase; PAL – phenylalanine ammonia lyase; ns - not significant.

(Fig. S1) were also evaluated to verify whether they were affected by mild salt stresses. The phenolic compounds identified included gallic acid, (+)-catechin, *p*-hydroxycinnamic acid, caffeic acid, *p*-coumaric acid and ellagic acid (Table 4). The most abundant phenolic acid in fruit was gallic acid, while the least abundant was the *p*-coumaric acid. The increase of total phenolic content in L2 was most likely related to the increase in the content of (+)-catechin (Table 4); the only phenolic compound evaluated that was improved in fruits under mild salt stress. Fauconneau et al. (1997) showed that catechins and epicatechins have strong antioxidant properties; therefore, the increase in this phenolic acid implies an improvement on the functional properties of strawberry fruit. Moreover, recent studies have demonstrated that (+)-catechins are important in plant stress tolerance. For example, the application of (+)-catechin conferred salinity tolerance to sweet pepper seedlings (Yiu, Tseng, Liu, & Kuo, 2012). It seems that this phenolic acid may also play a role in the stress response of strawberry plants.

Although the content of (+)-catechin was higher in L1 and L2 than the control group, the accumulation of gallic acid and caffeic acid was reduced in fruits from these treatments (Table 4), with the latter two compounds having a 98% positive correlation with each other (Table 6). L2 plants also had lower levels of *p*-coumaric acid compared to the other treatments. These data indicate that a balance of individual phenolic compounds must be achieved to confer defense against salt stress in strawberry.

Interestingly, the increased content of anthocyanins in L1 was accompanied by an increased content of sucrose (Table 3). Sucrose is a strong determinant of strawberry fruit quality because it is related to fruit sweetness and also indicates maturation progression (Gapper, McQuinn, & Giovannoni, 2013).

3.4. Mild salt stress levels have dose-dependent responses

In addition to their effects on biomass production and

Table 4
Content of phenolic compounds in strawberry fruit under mild salt stress.

Treatments	Gallic acid	(+)-Catechin	*p*-hydroxycinnamic acid	Caffeic acid	*p*-coumaric acid	Ellagic acid
	Fruits (g/kg)					
C	3.35 ± 0.01a	1.64 ± 0.09b	0.32 ± 0.11 ns	0.11 ± 0.001a	0.017 ± 0.001a	0.319 ± 0.04 ns
L1	2.96 ± 0.02b	1.70 ± 0.05a	0.34 ± 0.02	0.035 ± 0.002b	0.016 ± 0.001a	0.414 ± 0.08
L2	2.86 ± 0.02c	1.73 ± 0.02a	0.31 ± 0.02	0.037 ± 0.004b	0.009 ± 0.001b	0.304 ± 0.09

Strawberry plants (Camarosa cv.) were subjected to the application of distilled water (C treatment), 40 mmol/L NaCl (L1 treatment) or 80 mmol/L NaCl (L2 treatment). The means of four replicates ± SD are shown. All analysis were performed on dry weight. The content of each phenolic compound was compared between the three treatments. Therefore, different letters indicate significant differences ($P < 0.05$, Tukey's test) between treatments within a single column. ns - not significant.

Table 5
Gene expression analysis in strawberry fruit under mild salt stress.

Genes	C	L1	L2
Phenylpropanoid related genes ($2^{-\Delta\Delta Cq}$)			
FAL	1 ± 0.18b	1.36 ± 0.15b	3.20 ± 0.23a
C4H	1 ± 0.20a	0.70 ± 0.16ab	0.56 ± 0.12b
4CL	1 ± 0.26b	1.54 ± 0.24b	4.70 ± 0.52a
CHS	1 ± 0.11b	0.69 ± 0.10b	4.48 ± 0.45a
F3H	1 ± 0.04b	0.11 ± 0.10c	2.65 ± 0.77a
FLS	1 ± 0.03b	1.10 ± 0.21b	2.25 ± 0.46a
UFGT	1 ± 0.05b	0.48 ± 0.09b	6.63 ± 0.64a
ANS	1 ± 0.07b	0.39 ± 0.06c	1.81 ± 0.17a
LAR	1 ± 0.17b	1.59 ± 0.38a	1.26 ± 0.16b
ANR	1 ± 0.15a	0.45 ± 0.04b	0.54 ± 0.09b
Cell wall dissamble related genes ($2^{-\Delta\Delta Cq}$)			
EXP1	1 ± 0.32b	0.64 ± 0.25b	7.12 ± 1.30a
EXP2	1 ± 0.11 ns	1.29 ± 0.15	1.16 ± 0.14
EXP5	1 ± 0.24 ns	1.09 ± 0.05	1.39 ± 0.22
PME	1 ± 0.06 b	1.10 ± 0.26b	3.48 ± 0.63a
PG	1 ± 0.25b	7.18 ± 0.74a	8.34 ± 0.57a
Abscisic acid related genes ($2^{-\Delta\Delta Cq}$)			
NCED1	1 ± 0.05b	1.21 ± 0.12b	1.88 ± 0.14a
ASR	1 ± 0.19b	1.36 ± 0.20b	4.11 ± 0.35a
BG3	1 ± 0.23b	1.48 ± 0.11a	1.91 ± 0.20a

Strawberry plants Camarosa cv. were subjected to the application of distilled water (C), 40 mmol/L NaCl (L1) or 80 mmol/L NaCl (L2). Total RNA was isolated from fully-red fruit from the second sampling time according to Fig. 1 and used for RT-qPCR analysis. Genes related to the synthesis of compounds from the phenylpropanoid and flavonoid pathways, cell wall disassembly, and ABA metabolism and signaling were evaluated. The means of four replicates ± SD are shown. The control treatment (C) was used as a reference sample for gene expression analysis calculated according to the $2^{-\Delta\Delta CT}$ method. Different letters indicate significant differences ($P < 0.05$), according to Tukey's test. *PAL* (phenylalanine ammonia lyase), *C4H* (cinnamate 4-hydroxylase), *4CL* (ρ-coumarate ligase), *CHS* (chalcone synthase), *F3H* (flavanone 3-hydroxylase), *FLS* (flavonol synthase), *UFGT* (UDP flavonoid glycosyl transferase), *ANS* (anthocyanidin synthase), *LAR* (leucoanthocyanidin reductase), *ANR* (anthocyanidin reductase), *EXP1* (expansin 1), *EXP2* (expansin 2), *EXP5* (expansin 5), *PG* (polygalacturonase), *PME* (pectin methylesterase), *NCED1* (9-cis-epoxycarotenoid dioxygenase 1), *BG3* (beta-glucosidase 3), *ASR* (ABA stress and ripening induced).

photosynthetic rates, L1 and L2 also differed in several other variables evaluated. Because of the higher level of salt applied, the availability of water to be taken up by plants was affected in L2. These plants therefore showed the typical osmotic stress response of increased root weight (Table 1) because of the necessity of searching for available water at longer distances. A similar response has been observed in broccoli cultivated under water stress (Cogo et al., 2011). Moreover, the salt stress from L2 resulted in the induction of PAL activity and the increased accumulation of phenolic compounds in the fruit (Table 3). The L1 treatment did not induce root growth but increased the content of anthocyanins, sucrose and the activity of oxidant enzymes such as PO and PPO (Table 3). Therefore, plants subjected to both levels of salt stress showed specific defense responses, according to the salt level. The effects observed in L1 are most likely related to the ionic stress inflicted by the increased level of Na and Cl in the soil, while the effects in L2 were related to the association of ionic and osmotic stresses.

3.5. Mild salt stress affected the expression of genes related to quality traits

The effect of mild salt stress on the increased content of anthocyanins and phenolic compounds was directly related to the upregulation of key genes associated with their accumulation in strawberry fruit (Table 5). Anthocyanins and phenolic compounds are synthesized via the phenylpropanoid and flavonoid pathways (Fig. S1). The first step in the phenylpropanoid pathway is catalyzed by the enzyme phenylalanine ammonia-lyase (PAL), followed by serial reactions that lead to the production of different types of phenolic compounds. The induction of transcript levels occurs at the beginning of this metabolic pathway, maximizing the overall biosynthetic flux, by increasing *PAL* expression in L2 (Table 5). Because we have designed primers for the amplification of all five known *PAL* genes simultaneously, this expression represents the overall expression of PAL. The increased expression of *PAL* was also accompanied by higher activity of this enzyme (r = 0.97) and higher contents of phenolic compounds (r = 0.99), indicating a close relationship between *PAL* transcription, PAL activity and the content of phenolic compounds in those fruits (Table 6). This result is in agreement with previous studies of abiotic stress response (Fock-Bastide et al., 2014).

Although *PAL* expression had a 99.9% positive correlation ($P < 0.05$ by Pearson's test) with total phenolic content (Table 6), other rate-limiting enzymatic steps might also significantly influence the composition of phenolic compounds in fruit under mild salt stress. Therefore, the transcriptional level of nine other enzymes from this pathway were also evaluated. For this purpose, primers were designed for the amplification of one gene copy encoding each of the enzymes, according to the access number indicated in the Supplementary Table.

The genes *4CL* (related to the production of *p*-coumaroyl CoA), *CHS* (related to the production of chalcones), *F3H* (related to the synthesis of dehydroflavonols), *FLS* (related to the synthesis of flavonols), *ANS* (related to the synthesis of anthocyanidins), and *UFGT* (associated with the glycosylation of anthocyanins and flavonols) were upregulated only in L2. Most of these genes correlated positively with each other, and showed positive correlation with total phenolic compounds (Table 6), suggesting that they are key genes for the production of phenolic compounds under the osmotic stress inflicted by L2. In contrast, *ANR* (related to the conversion of anthocyanidins to (−)-epicatechins) and *C4H* (related to the synthesis of coumaric acid) were downregulated; they did not correlate with any of the above genes, and negatively correlated with (+)-catechin (r = −0.93 and −0.99, respectively). Therefore, there was a preferred induction of the synthesis of chalcones (*4CL*, followed by *CHS*), dihydroflavonols (*F3H*), flavonols (*FLS/UFGT*), and anthocyanidins (*ANS*) in L2, rather than *p*-coumaric acid (*C4H*) and (−)-epicatechins in general (*ANR*).

Although the content of anthocyanins increased in L1, *F3H* and *ANS* (genes associated with the synthesis of anthocyanin precursors) were downregulated. Only *LAR*, from the phenylpropanoid and flavonoid pathways, was upregulated, corresponding with the increased amounts of (+)-catechins. Therefore, (+)-catechin is one of the main compounds from these pathways to be produced, even under low levels of salt stress, as a defense response. Additionally, fruits from L2 showed upregulation of expansin genes, especially *EXP1*, suggesting a higher polysaccharide solubilization (Harrison, Mason, & Manning, 2001), as well as upregulation of *PG* and *PME* (Table 5), which are known to be involved in fruit softening (Vicente, Costa, Martinez, Chaves, & Civello, 2005). According to Ripoll et al. (2014), osmotic stress may modify expansin expression by decreasing cell turgor and water potential, enabling water to enter the cell and stimulate expansion. Fry, Dumville, and Miller (2001) supported the hypothesis that ROS participates in fruit softening. Cell wall disassembly caused by expansions, polygalacturonase, polymethylesterase, among others, is a major contributor to fruit texture during fruit ripening (Gapper et al., 2013). Therefore, the results of the present study suggest that mild salt stresses may affect several processes involved in the maturation of strawberry fruits, including accumulation of pigments and sugars as well as modification of the expression of cell wall related genes. Although all fruits used in the analysis were sampled when the fully red stage had been reached (according to Jia et al. [2011]), it appears that fruits from the mild salt stress

Table 6
Pearson's correlation matrix gene expression, enzyme activity and the content of compounds related to the phenylpropanoid and flavonoid pathways.

	Anthoc.	Ant. act.	Tot. Phen.	PAL act.	PAL gene	C4H gene	4CL gene	CHS gene	F3H gene	FLS gene	UFGT gene	ANS gene	LAR gene	ANR gene	Gal. acid	Catechin	Hyd. acid	Caff. acid	Coum. acid	Ellag. acid
Anthoc.	—																			
Ant. act.	0.01	—																		
Tot. Phen.	0.06	1.00	—																	
PAL act.	0.23	0.98	0.99	—																
PAL gene	0.00	1.00	1.00	0.97	—															
C4H gene	-0.53	-0.86	-0.88	-0.95	-0.85	—														
4CL gene	0.07	1.00	1.00	0.99	1.00	-0.88	—													
CHS gene	-0.21	0.97	0.96	0.90	0.98	-0.72	0.96	—												
F3H gene	-0.46	0.88	0.86	0.76	0.89	-0.51	0.85	0.96	—											
FLS gene	-0.06	1.00	0.99	0.96	1.00	-0.81	0.99	0.99	0.91	—										
UFGT gene	-0.20	0.98	0.96	0.91	0.98	-0.72	0.96	1.00	0.96	0.99	—									
ANS gene	-0.61	0.78	0.75	0.63	0.79	-0.35	0.75	0.90	0.98	0.83	0.90	—								
LAR gene	1.00	0.00	0.05	0.21	-0.01	-0.52	0.06	-0.23	-0.47	-0.08	-0.22	-0.62	—							
ANR gene	-0.83	-0.56	-0.60	-0.73	-0.55	0.91	-0.61	-0.36	-0.10	-0.50	-0.37	0.07	-0.83	—						
Gal. acid	-0.66	-0.76	-0.79	-0.88	-0.75	0.99	-0.80	-0.59	-0.36	-0.71	-0.60	-0.19	-0.65	0.96	—					
Catechin	0.58	0.82	0.85	0.93	0.81	-1.00	0.85	0.67	0.45	0.78	0.68	0.29	0.57	-0.93	-0.99	—				
Hyd. acid	0.57	-0.81	-0.78	-0.67	-0.82	0.39	-0.78	-0.92	-0.99	-0.85	-0.92	-1.00	0.59	-0.03	0.23	-0.33	—			
Caff. acid	-0.81	-0.60	-0.64	-0.76	-0.59	0.93	-0.64	-0.40	-0.15	-0.54	-0.41	0.03	-0.80	1.00	0.98	-0.95	0.02	—		
Coum. acid	0.03	-1.00	-1.00	-0.97	-1.00	0.83	-0.99	-0.98	-0.90	-1.00	-0.98	-0.81	0.04	0.53	0.73	-0.80	0.84	0.56	—	
Ellag. acid	0.85	0.53	0.57	0.70	0.52	-0.89	0.58	0.33	0.07	0.47	0.33	-0.11	0.85	-1.00	-0.95	0.92	0.07	-1.00	-0.49	—

Black-filled numbers indicate $P < 0.05$, and gray-filled numbers indicate $P < 0.1$.

Anthoc. – anthocyanins; Ant. Act. – Total antioxidant activity; Tot. Phen. – Total phenolic compounds; PAL act. – PAL activity; Gal. acid – gallic acid; Catechin – (+)-catechin; Hyd. Acid –*p*-hydroxycinamic acid; Caff. acid – caffeic acid; Coum. acid – *p*-coumaric acid; Ellag. acid – ellagic acid.

treatments show an advanced maturation stage. However, whether mild salt stress results in the acceleration of fruit ripening remains to be investigated. *Mild salt stress affects the expression of ABA-related genes.*

Abscisic acid (ABA) plays a major role in the induction of fruit ripening, as a critical factor for anthocyanin and sucrose accumulation, as well as loss of firmness (Jia et al., 2011). Because the mild salt stress evaluated in the present study induced the accumulation of anthocyanins and sucrose and induced the expression of genes associated with loss of firmness, we also evaluated the expression of abscisic acid-related genes to evaluate whether this phytohormone plays a role during mild salt stress response. ABA levels are increased via two biosynthetic mechanisms. One mechanism involves *de novo* biosynthesis of ABA, where NCED appears to be the key enzyme. In strawberry, it was observed that *FaNCED1*-RNAi downregulates ABA biosynthesis and inhibits fruit ripening (Jia et al., 2011). The other mechanism involves the one-step hydrolysis of ABA-glucose ester to release active ABA by BG (β-glucosidase). In strawberry fruit, *FaBG3* expression correlated well with changes in ABA levels, and *FaBG3*-RNAi downregulates the expression of a series of genes related to ripening and fruit quality, including genes involved in cell-wall catabolism, the anthocyanin synthesis pathway, aroma and sugar metabolism (Li, Ji, Luo, Wang, & Leng, 2013), resulting in transgenic fruits that do not fully ripen. In the present study, both ripening-related ABA genes, *NCED1* and *BG3,* were upregulated under mild salt stress (Table 5), suggesting a relationship with the induction of genes from the phenylpropanoid and flavonoid pathways. Interestingly, in a recent study (Li, Luo, Mou, Mao & Ying, 2015), strawberry fruits previously treated with ABA showed increased expression of the same genes associated with the synthesis of anthocyanins that were upregulated in the present study, and they also showed downregulation of *C4H*. Therefore, mild salt stress and exogenous application of ABA have similar effects on the expression of anthocyanins related-genes, and *C4H* may not act as a rate-limiting enzyme in this pathway.

In addition to the induction effect of mild salt stress on the expression of *NCED1* and *BG3*, the same behavior was observed for the expression of *ASR* (Table 5). ASR proteins are involved in fruit ripening and act as a downstream component of a common transduction pathway for sugar and ABA signals in strawberry fruit ripening (Chen et al., 2011). Altogether, these results suggest that the effect of mild salt stress on the maturation of strawberry fruits may occur through an ABA-dependent mechanism.

4. Conclusions

Mild salt stresses (40 and 80 mmol/L NaCl) influence molecular, biochemical and physiological responses of strawberry plants. Overall, the mild salt stress did not affect fruit yield; however, the lower level of salt stress positively affected photosynthesis, resulting in increased vegetative growth, and improved the content of anthocyanins and sucrose, while the higher level of salt stress induced root growth and the accumulation of phenolic compounds. Therefore, application of mild salt stress may be effective as a biofortification effort. These effects were accompanied by the upregulation of several genes from the phenylpropanoid and flavonoid pathways, as well as genes related to cell wall disassembly and ABA-related genes, suggesting that mild salt stress induces maturation of strawberry fruits via an ABA-dependent mechanism. Future studies of fruits cultivated under mild salt stress will elucidate whether this effect results in acceleration of fruit ripening and postharvest fruit quality changes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.lwt.2016.07.001.

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