Increasing Ascorbic Acid Content and Salinity Tolerance of Cherry Tomato Plants by Suppressed Expression of the Ascorbate Oxidase Gene

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HAL Id: hal-02620117

https://hal.inrae.fr/hal-02620117

Submitted on 25 May 2020

Increasing Ascorbic Acid Content and Salinity Tolerance of Cherry Tomato Plants by Suppressed Expression of the Ascorbate Oxidase Gene

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Abstract: Ascorbic acid is considered to be one of the most important antioxidants in plants and plays a vital role in the adaptation of plants to unfavorable conditions. In the present study, an ascorbate oxidase gene (Solyc04g054690) was over-expressed in cherry tomato cv. West Virginia 106 lines and compared with previously studied RNAi silenced ascorbate oxidase lines. Two lines with lower ascorbate oxidase activity (AO−15 and AO−42), two lines with elevated activity (AO+14 and AO+16), and the non-transgenic line (WVa106) were grown and irrigated with 75 mM and 150 mM NaCl in 2015 and 2016. Growth, yield, and chemical composition of the lines under salinity stress were evaluated. Lines with lower ascorbate oxidase activity resulted in higher plant growth parameters (plant height, leaf number, flower, and cluster number in 2015 and stem diameter and flower number in 2016), and improved fruit quality (firmness in 2016 and soluble solid content in 2015) and total yield per plant under salinity stress over both years. In addition, we show that ascorbic acid, lycopene, and carotene contents of fruits were higher in lines with lower ascorbate oxidase activity compared to lines with elevated activity and the non-transgenic line under conditions of moderate and high salinity in both years.

Keywords: vitamin C; NaCl; salt; GMO; *Solanum lycopersicum* Mill

1. Introduction

Salinity is a severe global problem limiting the growth and productivity of most agricultural crops. The percentage of affected land will increase in the coming few decades due to a decrease in the quantity and quality of irrigation water and global climate change. Most horticultural crops are sensitive to salinity caused by water and/or soil salinity. There are two principal strategies for controlling salinity: Establishing new irrigation and drainage systems or generating plants that are resistant to salinity. It has been shown that salinity stress during flowering negatively affects tomato plant yield by decreasing the number of fruits [1]. Plant response to salinity stress depends on the duration of exposure and salt levels. The response ranged from retarding growth and reducing yield under moderate salt levels to plant death under severe conditions [2]. For some crops, such as tomato, fruit quality can be improved under low or moderate salinity [3].

Tomato (*Solanum lycopersicum*) is considered to be one of the most important vegetable crops in the world. Tomato particularly attracts attention because the fruit contains considerable levels of vitamins, minerals, and antioxidants, which can prevent the development of various types of

cancer, including prostate, colon, and breast cancers [4]. Tomato fruits have a considerable amount of ascorbic acid (AA) (or vitamin C), which ranged from 84 to 590 mg/kg [5,6]. In addition, nearly one hundred grams of tomato can provide 40% of the recommended amount of AA for an adult's daily nutritional requirements. AA is considered to be one of the most abundant antioxidants found in the plant, including the apoplast [7]. There are several factors affecting AA levels in plants, such as the genetic background, environmental conditions, seasons, and abiotic stress, such as drought and salinity [8]. Previous work showed that application of exogenous AA leads to increased salt tolerance in tomato [2]. It was reported that breeders should be developing horticultural crops with high ascorbic acid content [9]. In plants, ascorbic acid is oxidized by ascorbate oxidase (AO) (an apoplastic enzyme) to mono-dehydroascorbate (MDHA) [10].

The activity of AO plays a role in regulating the redox state (the ratio of reduced to total AA) of the apoplast [11]. A positive relationship has been found between the level of AO activity and abiotic stress tolerance. AO is also highly expressed in fruits and roots of tomato [12].

Under-expression of the AO enzyme often leads to increased abiotic stress tolerance, such as to drought in tomato [11] and to salt in Arabidopsis and tobacco [13]. By contrast, the over-expression of AO leads to increased sensitivity to stress [7].

In a previous study [11], West Virginia 106 cherry tomato cotyledons were transformed to create two RNAi silenced lines (AO−15 and AO−42) with lower AO activity. In this work, the authors found that the yield of cherry tomatoes was increased in lines under-expressing the AO enzyme in plants under drought stress conditions compared to wild-type. In the current study, we evaluated the effect of changes in AO activity under conditions of saline stress. Transgenic lines and controls were grown under two salinity levels (moderate and high). Plant growth, yield, and fruit quality for the lines were also evaluated.

2. Materials and Methods

2.1. Development of Cherry Tomato Lines with Modified Ascorbate Oxidase Activity

Previous work [11] generated independent ascorbate oxidase RNAi silenced lines (Solyc04g054690, named AO−15 and AO−42); these lines had both lower ascorbate oxidase transcript and lower ascorbate oxidase activity compared to wild-type (data presented in Figure 2 of the cited published article). Over-expressing lines were generated for the experiments presented in this paper by cloning the same ascorbate oxidase cDNA fragment, Solyc04g054690, amplified from tomato first strand cDNA using the primers:

z2879F: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTccaacatggttgagcat-g

and z2879R: 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTtttaaggcctgtggaacc-ttttac.

The cDNA fragment was cloned into the Gateway vector pDONR201 (BP reaction), checked by sequencing to verify the absence of errors in the coding sequence, and subsequently transferred by an LR reaction in the Gateway vector pK7WG2D. WVa106 tomato cotyledons were genetically transformed based on a previously described method [14]. Following transformation, seven independent T0 lines were generated and lines were chosen based on their increase in ascorbate oxidase activity in plantlets. Two lines were selected (AO+14 and AO+16), which had an increase in AO activity compared to the wild-type (WT) (Figure 1). Ascorbate oxidase activity was measured according to Garchery et al. [11]. One of the chosen lines (AO+14) was tested for ascorbate oxidase gene expression by qPCR and showed at least a ten-fold over-expression of the gene relative to wild-type expression levels under normal and salt conditions.
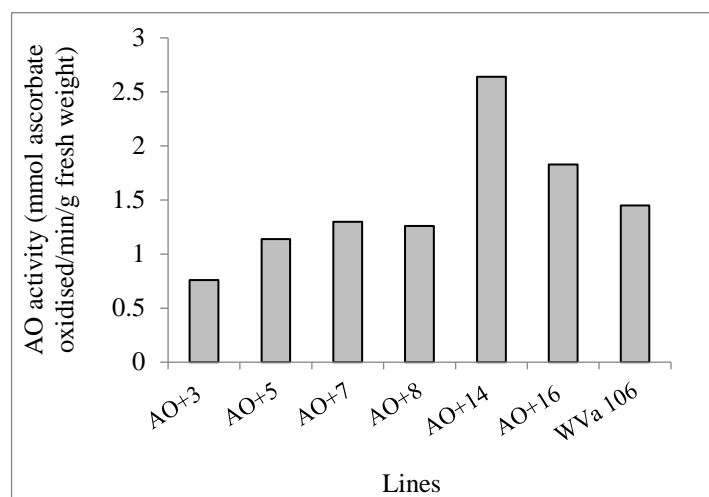
Figure 1. Ascorbate oxidase activity of primary transformants. Activity was measured in leaf tissue from individual primary transformants and expressed as millimoles of ascorbate oxidized at 265 nm per minute per gram of fresh weight of tissue.

2.2. Growth Conditions

Tomato plants (*Solanum lycopersicum* var. cerasiforme) variety West Virginia 106 (WVa106) were grown in a plastic greenhouse in 6 L black plastic pots filled with a 1:1 mixture of peat-moss and vermiculite in the 2015/2016 and 2016/2017 winter seasons at the Agricultural Experimental Station, Cairo University, Giza, Egypt. Nutrients in kg ha^{-1} (315 N, 225 P, and 450 K) required by tomato plants were supplied using drip irrigation. Disease and pest control were applied in accordance with needs and according to commercial practices. Lateral shoots were pruned as they appeared. The average temperature during plant growth was 27/20 °C (day/night), and mean relative humidity was 70–80%. The average photon flux density was 800–1000 µmol m^{-2} s^{-2} during the growing season.

2.3. Salinity Treatments

Fifteen days after transplantation, the saline treatments were applied with nutrient solution containing 0, 75, and 150 mM NaCl (El-Nasr pharmaceutical chemical company, Obour, Egypt). The saline treatments were continued until the end of the experiment (155 days after seedling transplantation). The mean ECs of the nutrient solution (containing salt) for 0, 75, and 150 mM NaCl were 1.2, 7.5, and 14 dSm^{-1}. The experimental design was factorial with two factors (three salinity levels and five lines); a complete randomized block design was used for the treatments. Five replicates for every treatment were used.

2.4. Plant Growth and Yield Characteristics

Plant height, leaf number, stem diameter, chlorophyll content using a SPAD (soil-plant analyses development) meter (SPAD 502 Minolta Co, Osaka, Japan), cluster number, and flower number were measured 45 days after the starting salt treatment. Plant height was measured from the soil surface to the highest growing tip. The stem diameter was measured using an electronic caliper. Four SPAD readings were taken around the tomato leaf edges and the average was calculated. Full red color fruits were harvested weekly. Ten fruits per plant were randomly selected for measuring mean fruit weight and mean fruit diameter. Total fruit yield per plant was calculated.

2.5. Determination of Chlorophyll a and b, β-carotene, Lycopene, and Ascorbic Acid

Chlorophyll a (Chl a) and b (Chl b) were measured according to Strain and Svec [15]. Briefly, one gram of red-ripe fruit was extracted with 90% acetone. The extraction solution was kept in the

dark overnight at room temperature. The samples were read using a spectrophotometer at 663, 645, and 750 nm. Chlorophyll a and b concentrations were calculated using the following formulae:

Chl a (μg g^{-1}) = 11.64 × (A_{663}) − 2.16 × (A_{645})

Chl b (μg g^{-1}) = 20.97 × (A_{645}) − 3.94 × (A_{663})

Lycopene and β-carotene were measured according to Nagata and Yamashita [16]. One gram of tomato juice from red-ripe fruits was homogenized with the extraction solution (acetone–hexane, 4:6). The extraction was centrifuged (Centurion Scientific, model 42802, TA-3A, Chichester, UK) for 20 min at 4930.38 g. The optical density of the supernatant at 663, 645, 505, and 453 nm was measured using a spectrophotometer. Lycopene and β-carotene were calculated according to the following equations:

Lycopene (μg g^{-1}) = −0.0458A_{663} + 0.204A_{645} + 0.372A_{505} + 0.0806A_{453}

β-carotene (μg g^{-1}) = −0.216A_{663} − 1.22A_{645} − 0.304A_{505} + 0.452A_{453}

Ascorbic acid (AA) in red-ripe fruits was measured according to the Association of Official Analytical Chemistry 967.21 [17]. Ascorbic acid content was determined using a titrimetric method with 2,6-dichlorophenol indophenol, and the results were expressed as mg 100 g^{-1} FW.

2.6. Determination of Soluble Solid Content and Firmness

Soluble solid content (SSC) of tomato juice extract was measured using a digital refractometer (model PR101, Co. Ltd., Tokyo, Japan). A drop of the juice was placed on the lens and the reading was taken in degrees Brix (Bx°) expressed as a % of soluble solid content in the fruit. Distilled water was used for calibration, and the lens was washed twice between samples.

Firmness was determined in red-ripe fruits using a Force Gauge Model M4-200 (ELECTROMATIC Equipment Co., Inc. Cedarhurst, NY 11516 USA) with a 1-mm diameter flat probe. Firmness values of each cherry tomato were measured at three points of the equatorial region and expressed in Newtons. Five fruits per replicate were used to measure firmness.

2.7. Statistical Analysis

Data were statistically analyzed using a two-way ANOVA test to detect the main effects of saline stress on lines, and the interactions between these two factors at a probability level of $p < 0.05$ using MSTAT software (Michigan State University, East Lansing, MI, USA). The mean values ± standard error (SE) were compared using a Duncan test. Pearson correlations between ascorbic acid levels and plant or fruit phenotypes were carried out for data from individual years and individual plants using SPSS software (IBM, Armonk, NY, USA), (Table 1).

Table 1. R values for Pearson correlations between ascorbic acid levels and phenotypes of individual plants in 2015 and 2016.

Phenotype Correlated with AA	*R* Values	
	2015	**2016**
Plant height (cm)	0.663 **	0.694 **
Stem diameter (mm)	0.552 **	0.608 **
Leaf number	0.622 **	0.622 **
Cluster number	0.625 **	0.553 **
Flower number	0.565 **	0.672 **
Fruit weight (g)	0.603 **	0.543 **
Fruit diameter (mm)	0.455 **	0.429 **
Yield (g plant^{-1})	0.732 **	0.760 **
Lycopene (μg g^{-1} fw)	0.722 **	0.791 **
ß- carotene (μg g^{-1} fw)	0.647 **	0.608 **

** significant at $p \leq 0.01$ Pearson correlation.

3. Results

3.1. Plant Growth

Plant height was significantly different ($p < 0.05$) between lines and salinity treatments in both years (Figure 2A,B). Plant height decreased with increasing salinity level. In general, there were no significant differences in plant height between the lines under normal growth conditions. However, at 75 mM NaCl, AO−15, and AO−42 showed the highest plant height compared to control or the AO+14 and AO+16 lines in 2015.
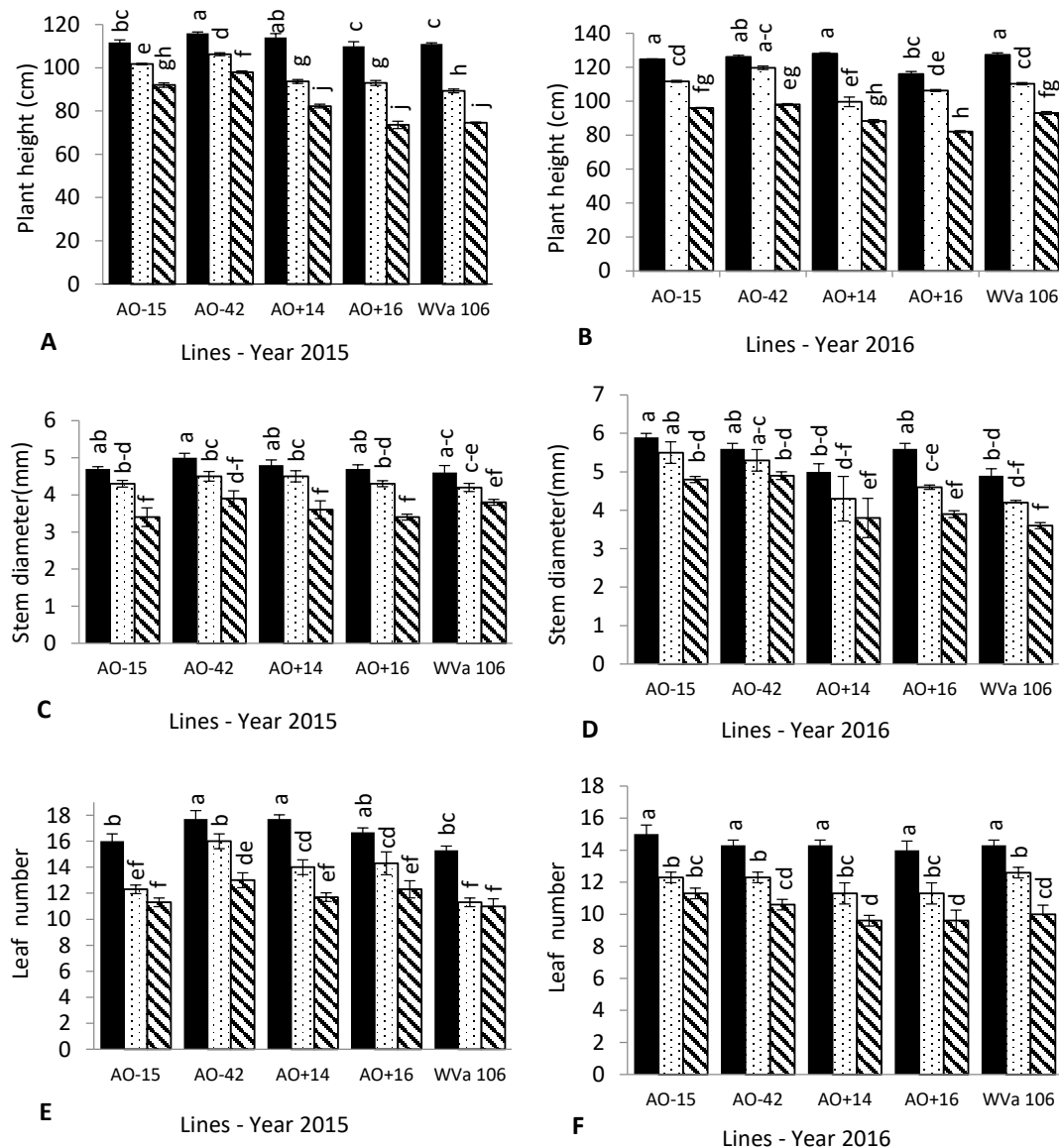

Figure 2. The effect of three salinity levels 0 mM (black bars), 75 mM (speckled bars), and 150 mM NaCl (striped bars) on cherry tomato lines. (**A**) Plant height in 2015, (**B**) plant height in 2016, (**C**) stem diameter in 2015, (**D**) stem diameter in 2016, (**E**) leaf number in 2015, and (**F**) leaf number in 2016. Results with the same superscript were not significantly different ($p < 0.05$) according to the classification obtained by the Duncan test.

The stem diameter also decreased with increasing salinity levels from normal conditions to 150 mM salt in 2015 and 2016 (Figure 2C,D). In 2015, there were no significance differences in stem diameter among lines either under normal conditions or under the two salinity levels. In 2016, no significant difference was found among lines under normal conditions. However, AO−15 and

AO−42 showed a larger stem diameter at 75 and 150 mM NaCl than the non-transgenic line or AO+14 and AO+16.

The interactions between salt levels and tested lines for leaf number in 2015 and 2016 are presented in Figure 2E,F, respectively. The leaf number trait decreased with increasing salinity levels in both years. In addition, there were no significant differences among lines under normal conditions in both years. In 2015, under moderate or high salinity, the highest significant leaf number was observed for the AO−42 line compared to the other under-expression line (AO−15), the two over-expression lines (AO+14 and AO+16), and the non-transgenic line (WVa106).

Data in Supplementary Figure S1A,B show that increasing salinity levels significantly increased the SPAD reading in both years. In 2015, no differences were observed in the SPAD reading for all lines under normal conditions or the two salinity levels. In 2016, the highest SPAD reading was recorded for the AO−42 line under 150 mM salt.

3.2. Cluster and Flower Number

The cluster number decreased with increasing salt levels in both years (Figure 3A,B). Under normal conditions, in 2015, there were no differences between lines. However, in 2015, under 150 mM salt, the highest cluster number values were recorded for AO−15 and AO−42 lines compared to the lines AO+14, AO+16, and the non-transgenic line. In 2016, there were no significant differences between AO−15 and AO−42 lines and the non-transgenic line (WVa106). The same trend was observed for the flower number trait in both years (Figure 3C,D), which decreased with increasing salt concentration. For this phenotype, results were clearer in terms of statistical differences in 2016, with AO silenced lines having more flowers under salt conditions (both 75 and 150 mM) than the wild-type or AO lines with elevated activity. However, the flower number under normal conditions was not statistically different for any of the lines. The same tendency was observed in 2015.
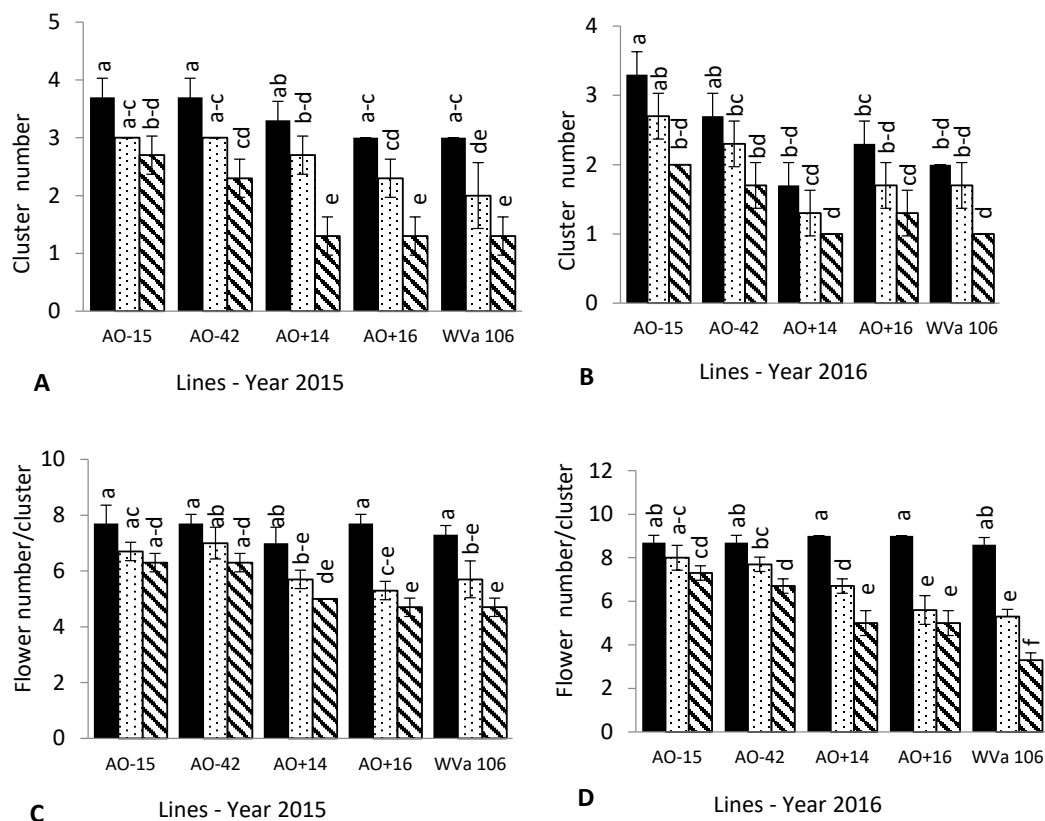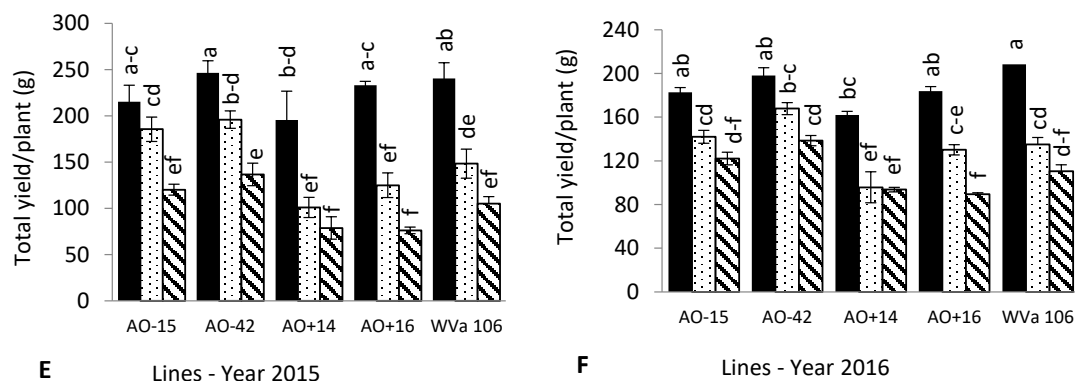

Figure 3. *Cont.*

Figure 3. The effect of three salinity levels 0 mM (black bars), 75 mM (speckled bars), and 150 mM NaCl (striped bars) on cherry tomato lines. (**A**) Cluster number in 2015, (**B**) cluster number in 2016, (**C**) flower numbers/ cluster in 2015, (**D**) flower number/cluster in 2016, (**E**) total yield/plant in 2015, and (**F**) total yield/plant in 2016. Results with the same superscript were not significantly different ($p < 0.05$) according to the classification obtained by the Duncan test.

3.3. Fruit Quality and Yield

Mean fruit weight and diameter decreased with increasing salt levels in both years of study (Supplementary Figure S1C–F). Mean fruit weight in 2015 did not change for any of the lines when lines were compared for a given condition (Supplementary Figure S1C). In 2016, no significant differences were found in terms of mean fruit weight for lines with lower or elevated AO activity at 75 or 150 mM salt and normal conditions (Figure 1D). However, the AO−42 line under the 150 mM salt condition showed increased fruit weight compared to the two lines with elevated AO activity and WVa106. Although the fruit diameter tended to decrease with increasing salt, no clear differences were found between the lines for a given condition (Supplementary Figure S1E,F).

Total yield per plant decreased significantly with increasing salt levels in both years (Figure 3E,F). Under normal conditions, no significant differences were observed between all lines. The highest total yield per plant was recorded for the AO−15 and AO−42 lines under salinity stress (75 and 150 mM salt), whereas the lowest yield values were recorded from AO+14, AO+16, and WVa106 in 2015 and from AO+14 and AO+16 in 2016. Under medium salt stress (75 mM) in 2015, yield in lines with lower AO activity was higher than in lines with elevated activity; this was also observed in 2016 between the two lines with lower AO activity and one of the lines with elevated AO activity.

3.4. Fruit Chemical Composition

Use of saline irrigation resulted in a decline in AA content in cherry tomato fruits (Figure 4A,B). Under control conditions, the AO−15 and AO−42 lines showed the highest AA content, followed by AO+16 and the non-transgenic line, AO+14 having the lowest AA levels. Under salt stress conditions (75 and 150 mM), AA in the AO−15 line was higher than in the AO+14 line and the non-transgenic line in both seasons.

The effect of salinity levels on chlorophyll a and b content in fruits of the different lines is presented in Supplementary Tables S1 and S2. In general, chlorophyll a and b content in cherry tomato fruits increased with increasing salinity levels, but this increase was not statistically different between the different lines. Chlorophyll a and b content did not change significantly for any of the lines under 0, 75, or 150 mM NaCl in either year.

No significant difference was found in terms of lycopene content between normal conditions and 75 mM salt in 2015 except for AO+14 and non-transgenic WVa106, whose lycopene content decreased at 75 mM salt (Table 2), while significant decreases were observed in 2016 with increasing salinity from 0 to 75 mM salt for all lines except AO−15. The decrease in lycopene content increased under 150 mM salt compared with 75 mM salt. In 2015 and 2016 for AO−15 and 2015 for AO−42, fruits had higher

lycopene content compared with the lines with elevated AO activity and the non-transgenic WVa106 line under 75 and 150 mM salt.
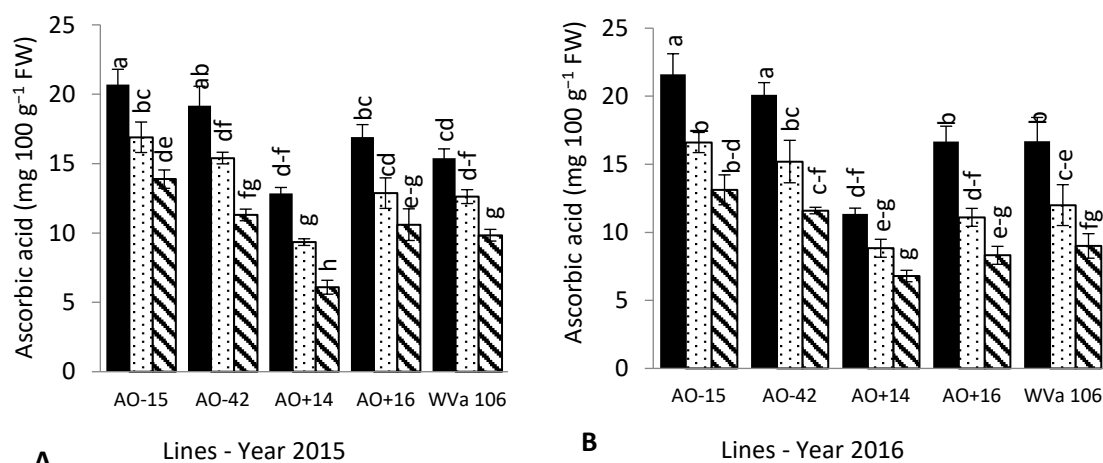

Figure 4. The effect of three salinity levels 0 mM (black bars), 75 mM (speckled bars), and 150 mM NaCl (striped bars) on ascorbic acid content of red-ripe fruit of cherry tomato lines. (**A**) Ascorbic acid (mg 100 g^{-1} FW) in 2015 and (**B**) ascorbic acid (mg 100 g^{-1} FW) in 2016. Results with the same superscript were not significantly different ($p < 0.05$) according to the classification obtained by the Duncan test.

Table 2. Lycopene content ($\mu g\ g^{-1}$ FW) of lines with low or elevated AO activity, and non-transgenic line of red-ripe cherry tomato fruits under two salinity levels (75 and 150 mM NaCl) in 2015 and 2016. Results with the same letter were not significantly different ($p < 0.05$) according to the classification obtained by the Duncan test.

Lines	2015			2016		
	0 mM	75 mM	150 mM	0 mM	75 mM	150 mM
AO−15	25.94 ± 1.11 a	25.10 ± 0.11 ab	21.44 ± 1.0 c–e	21.90 ± 0.75 ab	21.10 ± 0.59 a–c	20.51 ± 0.87 bc
AO−42	25.42 ± 0.45 a	25.32 ± 0.3 a	19.31 ± 0.81 ef	21.12 ± 0.32 a–c	17.25 ± 0.75 d	17.14 ± 0.44 d
AO+14	25.18 ± 1.2 ab	19.86 ± 0.95 de	15.10 ± 0.56 g	22.61 ± 0.84 a	17.78 ± 0.53 d	16.37 ± 0.15 de
AO+16	22.37 ± 1.14 b–d	19.91 ± 1.81 de	16.74 ± 0.21 fg	20.44 ± 0.37 bc	16.24 ± 0.23 de	16.65 ± 0.61 de
WVa106	23.25 ± 1.05 a–c	15.24 ± 1.02 g	14.71 ± 0.42 g	19.52 ± 0.37 c	16.59 ± 0.73 de	15.23 ± 0.55 e

Carotenoid content decreased with increasing concentrations of NaCl from normal conditions to 75 mM and 150 mM in 2015 and 2016 (Table 3). Both lines with lower activity AO−15 and AO−42 showed a significantly higher carotenoid content at 75 and 150 mM salt (in 2015) compared to AO+14, AO+16, and WVa106, while in 2016, the lines with lower AO activity had higher carotenoid content in fruit at 75 mM salt compared to the two lines with elevated AO and the non-transgenic line (WVa106).

Table 3. Carotenoid content ($\mu g\ g^{-1}$ FW) of lines with low or elevated AO activity and non-transgenic line of red-ripe cherry tomato fruits under two salinity levels (75 and 150 mM NaCl) in 2015 and 2016. Results with the same letter were not significantly different ($p < 0.05$) according to the classification obtained by the Duncan test.

Lines	2015			2016		
	0 mM	75 mM	150 mM	0 mM	75 mM	150 mM
AO−15	4.37 ± 0.31a	3.58 ± 0.81 ab	3.12 ± 0.42 ab	3.90 ± 0.43 ab	3.10 ± 0.18 b	2.00 ± 0.15 c
AO−42	3.62 ± 0.35 a	3.47 ± 0.65 ab	3.00 ± 0.46ab	4.20 ± 0.26 a	3.30 ± 0.27 ab	2.10 ± 0.27 c
AO+14	3.88 ± 0.56 a	1.66 ± 0.48 cd	1.62 ± 0.01 cd	3.60 ± 0.28 ab	2.00 ± 0.21 c	1.50 ± 0.21 c
AO+16	3.00 ± 0.53 a–c	1.95 ± 0.12 cd	1.71 ± 0.15 cd	3.40 ± 0.23 ab	1.80 ± 0.15 c	1.70 ± 0.12 c
WVa106	3.86 ± 0.82 a	1.58 ± 0.49 cd	1.08 ± 0.49 d	3.90 ± 0.42 ab	2.10 ± 0.20 c	1.09 ± 0.50 c

Table 4 shows that SSC increased with increasing salinity levels in 2015 and 2016. The AO−42 line, in 2015, showed a higher SSC value compared to non-transgenic WVa106 under 75 and 150 mM NaCl. Moreover, in 2016, the AO−42 line showed the higher SSC value compared to the lines with elevated AO activity and WVa106 under 75 mM NaCl.

Table 4. Soluble solid content (%) of lines with low or elevated AO activity and non-transgenic line of red-ripe cherry tomato fruits under two salinity levels (75 and 150 mM NaCl) in 2015 and 2016. Results with the same letter were not significantly different ($p < 0.05$) according to the classification obtained by the Duncan test.

Lines	2015			2016		
	0 mM	75 mM	150 mM	0 mM	75 mM	150 mM
AO−15	6.00 ± 0.57 ef	7.04 ± 0.28 b–d	7.76 ± 0.15 ab	6.33 ± 0.24 ef	6.70 ± 0.37 de	7.31 ± 0.25 cd
AO−42	7.00 ± 0.21 b–d	7.33 ± 0.17a–c	7.99 ± 0.14 a	6.72 ± 0.23 de	7.85 ± 0.37 a–c	8.46 ± 0.27 a
AO+14	6.26 ± 0.08 d–f	6.96 ± 0.27 b–d	7.43 ± 0.05 a–c	6.57 ± 0.08 e	6.97 ± 0.2 de	7.33 ± 0.08 b–d
AO+16	5.93 ± 0.16 ef	6.43 ± 0.06 de	6.93 ± 0.08 b–d	5.31 ± 0.16 g	6.30 ± 0.05 ef	8.07 ± 0. 2 ab
WVa106	5.6 ± 0.201 f	6.56 ± 0.24 de	7.01 ± 0.31 b–d	5.70 ± 0.14 fg	6.41 ± 0.24 e	7.71 ± 0.12 a–c

The firmness of cherry tomato fruits increased with increasing salinity levels (Table 5). Under control conditions and 75 mM NaCl, there was no significant difference among tested lines in both years of study. In 2016, the highest firmness values were recorded at 150 mM salt for the AO−15 and AO−42 lines.

Table 5. Firmness (N) of lines with low or elevated AO activity and non-transgenic line of red-ripe cherry tomato fruits under two salinity levels (75 and 150 mM NaCl) in 2015 and 2016. Results with the same letter were not significantly different ($p < 0.05$) according to the classification obtained by the Duncan test.

Lines	2015			2016		
	0 mM	75 mM	150 mM	0 mM	75 mM	150 mM
AO−15	0.43 ± 0.01 a–c	0.42 ± 0.01 a–c	0.51 ± 0.01 a	0.36 ± 0.01 f–h	0.41 ± 0.01 c–e	0.50 ± 0.02 ab
AO−42	0.31 ± 0.01 cd	0.41 ± 0.01 a–d	0.48 ± 0.01 ab	0.35 ± 0.01 fg	0.40 ± 0.02 c–f	0.53 ± 0.01 a
AO+14	0.36 ± 0.01 b–d	0.38 ± 0.09 a–d	0.41 ± 0.1 a–d	0.36 ± 0.05 gh	0.36 ± 0.03 ef	0.43 ± 0.01 cd
AO+16	0.28 ± 0.01 d	0.31 ± 0.01 cd	0.36 ± 0.01 b–d	0.28 ± 0.04 h	0.35 ± 0.01 fg	0.45 ± 0.02 c
WVa106	0.35 ± 0.02 b–d	0.41 ± 0.01 a–d	0.45 ± 0.02 a–c	0.28 ± 0.01 h	0.38 ± 0.03 d–f	0.38 ± 0.01 d–f

4. Discussion

One of the most negative effects for plants after exposure to salinity is the reduction of plant biomass and plant growth. In our experiment, a reduction in plant height, stem diameter, and number of leaves was recorded following salinity treatments. In a previous study, increasing salt levels to 150 mM or above significantly decreased most growth parameters of tomato plants, including plant length and leaf number [18]. The adverse effects of saline stress on plant growth might be due to reduced photosynthesis [19] and the presence of Na^+ and Cl^- ions, which create an ionic imbalance [20]. In some plants, it has been reported that salt tolerance was improved, possibly due to increases in total AA content and dehydroascorbate reductase activity [21]. In our study, decreased ascorbate oxidase activity resulted in increases in AA content. We also found that under salt conditions, lines with lower AO activity were more resistant to salinity compared to the lines with higher AO activity or the non-transgenic line.

Plant cell growth is a combined process of cell division and cell elongation. Cell elongation occurs after cell division [22]. Previous articles have suggested that AA might be involved in plant cell growth processes [23]. Our results are in harmony with previous reports on the effect of AA on plant growth. Yamamoto et al. [13] reported that under-expression of the AO gene increased salt tolerance during the vegetative growth stage in Arabidopsis plants. The explanation for our results might be due to the fact that there is a higher AA content in lines with lower AO activity compared to those of the

non-transgenic line and lines with elevated activity [24]. In addition, plant height, stem diameter leaf number, and in particular yield were significantly positively correlated with AA in both seasons, which supported our results (Table 1).

Significant increases were observed in SPAD readings, with increasing salt concentrations in both years of our study. The increase in SPAD reading with increasing salinity levels might be due to reduced leaf size and increased leaf thickness resulting in higher chlorophyll density [25]. However, no differences were observed in SPAD readings among all tested lines.

It has been reported that the cluster number per plant decreased with increasing electrical conductivity levels (our results [26]). Our results also indicated that cluster numbers in 2015 and flower numbers in both seasons were significantly ($p < 0.05$) higher in lines with lower AO activity under salinity conditions. Previous work indicated that AA plays an important role in the biosynthesis of some plant hormones including gibberellin [27]. It was also recorded that exogenous application of AA increased the number of flowers per plant [28].

Salt stress (75 and 150 mM NaCl) significantly decreased mean fruit weight and fruit diameter in all lines compared to normal conditions in both years. This finding is in accordance with Assimakopoulou et al. [26], who reported that average fruit weight and diameter decreased when water electrical conductivity increased from 75 to 150 mM in some cherry tomato cultivars. In our study, cherry tomato fruit weight and diameter were higher under saline conditions (150 mM salt) for the AO−42 line compared to lines with elevated AO activity in 2016 and 2015, respectively. Additionally, a significant correlation was reported in our study between AA content in fruits and fruit weight and diameter. These findings are in agreement with those of Garchery et al. [11], who indicated that yield increased for the transgenic lines with lower AO enzyme activity compared to wild-type. This increase might be due to fruit sugar content in the symplastic space and an increased hexose:sucrose ratio in the apoplastic space in these lines.

In our experiment and that of Del Amor et al. [29], the highest yield per plant was recorded under conditions of normal irrigation. Our results confirmed that the line AO−42 had the highest total yield under 150 mM NaCl salinity stress in both seasons. A positive significant correlation was found between AA content and yield in both seasons. This result is in agreement with that of Garchery et al. [11], where increased fruit yield of cherry tomato was reported in AO RNAi lines. Interestingly, the lines with elevated AO activity are not easily distinguishable from the wild-type; this may be because the activity increases obtained were relatively low, possibly because an increase in AO activity has a detrimental effect on the plant.

One of the most important antioxidants in plants is ascorbic acid (AA), and this molecule is a major contributor to cellular redox state [30]. In our study, AA content of fruits decreased significantly with increasing salinity levels. In accordance with our result, it has been reported that AA and glutathione were significantly decreased in tomato cv. Naomi fruits irrigated with 6 dSm^{-1} [31]. Additionally, high NaCl concentrations in the nutrient solution resulted in lower AA content [32]. In addition, the high temperatures encountered during our experiment (28 and 26 °C in 2015 and 2016) could have inhibited the biosynthesis of AA [33].

We have reported in this study that the AA content of the AO−15 line increased significantly ($p < 0.05$) compared to lines with elevated activity and the non-transgenic line in both years under normal conditions, 75, and 150 mM salt. Furthermore, AA content showed significant correlations with tested parameters (Table 1). This correlation indicates that improvement of growth and yield could be due to increases in AA content. It has been previously mentioned that transgenic tomato plants with lower AO gene expression showed increased AA accumulation in tomato fruit [34].

The negative or positive effects of salinity on bioactive compounds in fruits and vegetables depend on various factors, such as concentrations and duration of salinity, the type of salt used, growing conditions, the crop type under study, other environmental conditions, and agricultural practices [35].

In this study, we found that lycopene content in tomato fruits decreased at high salinity levels. De Pascale et al. [36] reported increases in lycopene content in tomato fruit by irrigation with saline

solution at a rate of 4.4 dS m^{-1}. On the other hand, Serio et al. [31] did not find any changes in lycopene content associated with an increased electrical conductivity of the irrigation solution. The differences they found compared to our results may be due to the fact that carotenoid content was not only affected by saline stress but also by the genotypes and growing conditions [37]. Additionally, the high temperature of more than 29 °C during our experiment could be an extra factor, with the salinity stress, leading to reduced lycopene content [33]. In both seasons, the lines with lower AO activity showed the highest lycopene content values at 75 and 150 mM NaCl compared to lines with elevated activity and the non-transgenic line. This result could be due to the increase in AA content in these lines leading to increases in photosynthetic processes under salinity stress conditions, which could result in higher lycopene content [34]. Moreover, AA content showed a significant correlation with lycopene content in both seasons (Table 1). Moreover, these differences may be due to the higher temperature in 2015 compared to 2016.

Similarly to other studies, we obtained decreases in carotene content with increasing salinity from 75 to 150 mM NaCl. Ali and Ismail [38] found that the use of 100 mM NaCl in the nutrient solution resulted in a significant decrease in tomato fruit carotenoids. In addition, salinity levels higher than 4.4 dS m^{-1} decreased carotenoid content [36]. This result could be due to the negative effects of saline stress on carotenoid biosynthesis and the inhibition of lycopene accumulation [39]. Additionally, high concentrations of NaCl in nutrient solutions lead to lower carotene levels [32]. A significant positive correlation was also observed between AA content and lycopene content in our study. The relation between high AA content and carotene biosynthesis is unknown.

SSC content in tomato fruits is considered to be one of the main factors influencing tomato quality. Our findings showed that SSC increased with increasing salinity levels in both years and for all lines. It has previously been reported that 86 mM NaCl increased SSC content [40]. This result is in also accordance with a previous study [41]. Our result may be due to lower fruit water content, as irrigation with high salinity water leads to increases in percentage SSC [42]. The adaptation of tomato plants to salinity by increasing SSC in plant tissues could also be another explanation for this result. The results indicated that the AO−42 line had the highest significant SSC value compared to all other lines in 2015. This result could be partly due to the previously observed increase of tomato sugar content in AO RNAi lines [11].

Firmness increased significantly in all lines ($p < 0.05$) with increasing water salinity in 2016. The same trend was observed by El-Mogy et al. [3], which supports our results. Increases in firmness could either be due to salinity strengthening tomato skin resulting in increases in its thickness [43] or the presence of smaller cells with thicker walls in the pericarp of tomatoes grown under salinity conditions [44]. Our results indicated that the AO−42 line had the highest significant firmness value compared to all other lines in 2015. This result might be related to the increases of some chemical compositions in fruits, such as AA, carotenoids, and lycopene, as well as the SSC content, which were observed in this study.

5. Conclusions

In this study on cherry tomato cv. West Virginia 106, we used genetically modified lines with elevated or lower ascorbate oxidase, which were grown under 75 mM and 150 mM NaCl. Our results showed that lower ascorbate oxidase activity resulted in higher plant height in 2015, stem diameter in 2016, leaf numbers in 2015, and flower numbers and fruit yield in both years compared with lines with elevated activity and the non-transgenic line under salinity stress conditions. Moreover, AA, lycopene, carotene, and SSC contents of fruits were higher in lines with lower AO activity under salinity stress. A significant correlation was found between AA content and plant growth and yield, which supports our hypothesis that the manipulation of the ascorbate oxidase gene by breeding or other methods might be helpful for growing cherry tomato lines under salinity conditions.

Author Contributions: Data curation: M.M.E.-M. and R.G.S.; formal analysis: M.M.E.-M.; investigation: R.G.S.; methodology: K.F.A., M.M.E.-M., and C.G.; resources: C.G.; software: M.I.A.M. and C.G.; supervision: M.M.E.-M. and R.G.S.; visualization: K.F.A; writing—original draft: R.G.S.; writing—review and editing: R.G.S.

Conflicts of Interest: The authors declare no conflict of interest.

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