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Phenolic Metabolism in Grafted versus Nongrafted Cherry Tomatoes under the Influence of Water Stress

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ABSTRACT: Use of grafts using rootstocks capable of palliating the effects of water stress can be a possible solution to reduce yield losses. For response to stress, plants can induce the metabolism of phenylpropanoids. The aim of the present work is to determine the response of reciprocal grafts made between one tolerant cherry tomato cultivar, Zarina, and a more sensitive cultivar, Josefina. The analysis of the phenylpropanoids pathway was carried out both enzymatically and metabolically. DAHP synthase, shikimate dehydrogenase, phenylalanine ammonium-lyase, cinnamate 4-hydroxylase, and 4-coumarate CoA ligase activities were determined, and characteristic metabolites from the pathway were measured by means of HPLC-MS. Growth in the grafts *JosxZar* and *ZarxJos* was not appreciably affected by stress. *JosxZar* had increased concentrations of phenolic compounds after water stress. This could be correlated with the greater activity of synthesis enzymes as well as a decrease in phenol-degrading enzymes. Phenolic metabolism is more influenced by the aerial part, and therefore it is concluded that the capacity of inducing tolerance in rootstocks depends on the genotype of the shoot.

KEYWORDS: *Solanum lycopersicum*, quercetin, kaempferol, HPLC, phenolic compounds, shikimate pathway, grafting

INTRODUCTION

Water has become a scarce resource in many regions of the world, especially in arid and semiarid zones of the Mediterranean basin. Increased competition for water between agriculture, industry, and urban consumption brings into focus the need to improve irrigation practices in commercial plant production.[1] One possible solution to reduce yield losses and improve crop growth under water-deficit conditions involves the use of grafts using rootstocks capable of palliating the effects of this stress in the shoot.[1] Zones that produce grafted horticultural produces of great economic importance, including annuals of the family Solanaceae such as tomato, eggplant, and pepper, have in recent years begun to use plants that are resistant to soilborne diseases,[2] tolerant to environmental stress such as salinity and extreme temperatures,[3,4] and more efficient in water and nutrient uptake[5] and provide better fruit quality.[6] Today grafts are considered a rapid alternative to conventional reproduction, with the aim of boosting tolerance against environmental stress in crops.[7]

Plants have developed diverse mechanisms to combat damage caused by water stress. One biochemical factor involved in the response to stress is the metabolism of phenylpropanoids.[8] Plant resistance to biotic and abiotic stress is often regulated by the metabolism of phenolic compounds, so that greater activity of related enzymes and the accumulation of phenolic compounds have been correlated with resistance to these types of stress.[8] These compounds are generally synthesized by the shikimate pathway, using intermediates of carbohydrate metabolism.[9] The pathway begins with 3-deoxy-7-phosphoheptulonate synthase (DAHPS, EC 2.5.1.54), which is the key enzyme controlling the carbon flow toward phenolic metabolism. Another important enzyme in the pathway is phenylalanine ammonia-lyase (PAL, EC 4.3.1.24), which catalyzes the nonoxidative deamination of L-phenylalanine to form *trans*-cinnamic acid. This reaction is the first step in the biosynthesis of a great number of secondary products derived from phenylpropanoid in plants such as flavonoids and isoflavonoids, coumarins, lignins, hydroxycinnamic acid esters, and phenolic compounds.[10] In response to different types of environmental stress, increases have been found in PAL activity and in other enzymes of the phenylpropanoid pathway in tomato and lettuce plants.[4,11] On the other hand, some important differences were detected for the contents of phenolic compounds during the growing periods; kaempferol accumulates in the leaves of grafted and nongrafted watermelon plants and rootstocks.[8]

Given that tomato is one of the most important crops worldwide and that its production is concentrated in semiarid regions, where water stress is frequent, it is of great interest to ascertain whether grafting is a valid strategy to improve water-stress tolerance in this plant. In preliminary studies, we have observed that the cv. Zarina shows better water-stress tolerance than the cv. Josefina, which is more drought sensitive.[12] In this light, the aim of the present work is to determine the response of reciprocal grafts made between one tolerant cultivar, Zarina, and a more sensitive cultivar, Josefina, to moderate water stress, examining phenolic metabolism and the accumulation of its metabolites.

MATERIALS AND METHODS

Plant Material and Treatments. Two tomato (*Lycopersicon esculentum* Mill) cultivars, Zarina and Josefina, were used as scion and rootstock. The seeds of these cultivars were germinated and grown for

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30 days in a tray with wells (each well 3 cm × 3 cm × 10 cm) in the nursery Semillero Saliplant S.L. (Carchuna, Granada). Grafting was performed when seedlings had developed three to four true leaves. In the vermiculite trays used for germination, the seedlings were cut over the cotyledons, using the shoot as scion and the remaining plant part as rootstock. Grafts were made immediately after cutting the plants, and grafting clips were used to adhere the graft union. Self-grafted plants were included as controls. After grafting, seedlings were covered with a transparent plastic lid to maintain a high humidity level and to facilitate graft formation and were left in the dark for 24 h. The plastic was opened slightly every day to allow reduction in relative humidity, and it was removed 6 days after grafting. Afterward, ungrafted and grafted plants were transferred to a cultivation chamber at the Plant Physiology Department of the University of Granada under controlled conditions with a relative humidity of 50 ± 10% at 25 °C/15 °C (day/night) and a 16/8 h photoperiod with a photosynthetic photon-flux density (PPFD) of 350 μmol/m^2/s (LI-COR Inc., Lincoln, NE). Under these conditions, the plants grew in individual pots (25 cm upper diameter, 17 cm lower diameter, and 25 cm high) of 8 L in volume filled with a 1:1 perlite/vermiculite mixture. The complete nutrient solution used to grow the plant during the experiment was the same as described in a recent study.[12] The water-stress treatments began 45 days after germination and were maintained for 22 days. The control treatment received 100% field capacity (FC) irrigation, whereas moderate water stress corresponded to 50% FC. The experimental design was a randomized complete block with 12 treatments (Zarina ungrafted, Josefina ungrafted, Zarina self-grafted, Josefina self-grafted, *JosxZar*, and *ZarxJos* either well-watered (100% FC) or under water stress (50% FC)) arranged in individual pots with six plants per treatment (one plant per pot) and three replications each.

Relative Growth Rate (RGR) and Leaf Relative Water Content (LRWC). All plants were at the late vegetative stage when harvested. Leaves fully expanded (excluding petioles) and roots were harvested, frozen immediately in liquid N_2, and kept at −80 °C until used. To determine the RGR, leaves and roots from three plants per cultivar were sampled on day 45 after germination, immediately before the start of the water-stress treatment (T_i). The leaves and roots were lyophilized, and the dry weight (DW) was recorded as grams per plant. The remaining plants were sampled 67 days after germination (22 days of treatments, T_f). The relative growth rate was calculated from the increase in DW at the beginning and at the end of the water-stress treatment, using the equation $RGR = (\ln DW_f - \ln DW_i)/(T_f - T_i)$, where T is the time and the subscripts denote the final and initial samplings (i.e., days 0 and 22, respectively, after the water-stress treatment).[13]

LRWC was measured following the method of Barrs and Weatherley.[14]

Analysis of Phenolic Compounds by HPLC/UV-PAD/ESI-MS^n. For the identificaiton and characterization of phenolics, 0.1 g of lyophilized leaves was extracted with 1 mL of water/methanol (1:1) by sonication for 1 h, followed by overnight maceration and another sonication period (1 h). The resulting extract was centrifuged and filtered through a 0.45 μm PVDF membrane.

Chromatographic analyses were carried out on a Phenomenex reverse-phase column, 250 mm × 4.6 mm i.d., 5 μm, Li-Chrospher 100 RP-18, with a 4 mm × 4 mm i.d. guard column of the same material (Luna, Phenomenex). The mobile phase consisted of two solvents: water/acetic acid (1%) (A) and acetonitrile (B), starting with 5% B and using a gradient to obtain 50% at 30 min and 80% at 37 min. The flow rate was 1 mL/min and the injection volume, 20 μL. Spectroscopic data from all peaks were accumulated in the range of 200−400 nm, and chromatograms were recorded at 280, 320, and 360 nm. The HPLC/UV-PAD/ESI-MS^n analyses were carried out with an Agilent HPLC equipped with a PAD and mass spectrometer in series (Agilent Technologies, Waldbronn, Germany).

The mass spectrometer was an ion trap mass analyzer equipped with an electrospray ionization interface. The ionization conditions were adjusted at 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. The full-scan mass covered the range from m/z 100 to 1200. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 to 2 V. Mass spectrometry data were acquired in the negative ionization mode. MS^n was carried out on the most abundant fragment ion observed in the first-generation mass spectrum.

The identification of the peaks was obtained by analyzing the extracted-ion chromatograms of the ion current at m/z values corresponding to the $[M - H]^-$ ions of the individual investigated compounds, as well as their fragmentation. Quantification of the identified analytes was performed by HPLC-PDA detection using the external standard method with calibration graphs, as a function of concentration based on peak area, detected at the wavelength corresponding to their maximum absorbance.

Preparation of Enzyme Extract for Assay. For determination of 3-deoxy-7-phosphoheptulonate synthase (DAHPS, DS-Mn, DS-Co, EC 2.5.1.54) and PAL (EC 4.3.1.24) activities, whole fresh leaf was homogenized in 100 mM potassium phosphate buffer (pH 8.0) containing 1.4 mM 2-mercaptoethanol. The homogenate was centrifugated at 15000*g* for 15 min at 4 °C. The supernatant was passed through a Sephadex G-25 column (24 × 100 mm) previously equilibrated with the same buffer.

For determination of shikimate dehydrogenase (SKDH, EC 1.1.1.25) and polyphenol oxidase (PPO, EC 1.10.3.2) activities, whole fresh leaf was homogenized in 50 mM potassium phosphate buffer (pH 7.0). Homogenates were centrifuged at 15000*g* for 15 min at 4 °C.

For determination of cinnamate 4-hydroxylase (C4H, EC 1.14.13.11) fresh sample of leaf was homogenized in 200 mM potassium phosphate buffer (pH 7.5) containing 2 mM 2-mercaptoethanol. Homogenates were centrifuged at 10000*g* for 15 min at 4 °C.

For determination of 4-coumarate coenzyme A ligase (4CL, EC 6.2.1.12) the extract buffer was 0.05 M Tris-HCl (pH 8.8) containing 14 mM mercaptoethanol and 30% glycerol. Homogenates were centrifuged at 10000*g* for 15 min at 4 °C.

For determination of guaicol peroxidase (GPX, EC 1.11.1.9) the extract buffer was 50 mM Tris-HCl (pH 7.5) containing 5 mM mercaptoethanol, 2 mM DTT (dithiothreitol), 0.5 mM PMSF, and 2 mM EDTA-Na. Homogenates were centrifuged at 16500*g* for 30 min at 4 °C.

Enzyme Assay. DAHPS activity was assayed using a modified method.[15] The reaction mixture for DS-Mn was 50 mM K-Epps buffer (pH 8.0), 0.5 mM DTT, 0.5 mM $MnCl_2$, 3 mM PEP (phosphoenolpyruvate), and 0.6 mM E4P (erythrose-4-phosphate). The reaction mixture for DS-Co was 50 mM K-Epps buffer (pH 8.6), 10 mM $MgCl_2$, 3 mM PEP, and 3 mM E4P. DS-Mn and DS-Co reactions were initiated by the addition of enzyme extract to the reaction mixture, followed by incubation for 30 min for DS-Mn and for 20 min for DS-Co at 37 °C. The reaction was terminated by adding 25% trichloroacetic acid to the reaction mixture. For controls, 25% trichloroacetic acid was added to the mixture prior to the start of the reaction. After centrifugation (10000*g*, 15 min, 4 °C), supernatant was collected, to which 25 mM $NaIO_4$ containing 0.125 N H_2SO_4 was added. After incubation for 30 min at 37 °C, 2%$NaAsO_2$ containing 0.5 N HCl and thiobarbituric acid were added, followed by incubation for 10 min at 100 °C. The absorbance at 280 nm was then measured.

PAL activity was measured by a modified method.[16] The reaction mixture was 0.4 mL of 100 mM Tris-HCl buffer (pH 8.8), 0.2 mL of 40 mM phenylalanine, and 0.2 mL of enzyme extract. The reaction mixture was incubated for 30 min at 37 °C, and the reaction was terminated by adding 25% trichloroacetic acid. In the control of the PAL assay, the same amount of phenylalanine was added after termination.

Table 1. Influence of Moderate Water Stress on Dry Weight, RGR, and LRWC in Ungrafted, Grafted, and Self-Grafted Tomato Plants[a]

rootstock × scion/treatment	total biomass (g DW)	total RGR (mg/g/day)	radicular RGR (mg/g/day)	LRWC (%)
Zar ungrafted				
well-watered	7.06 ± 0.73 a	62.34 ± 5.03 a	38.56 ± 3.60 b	89.97 ± 1.00 a
water stress	6.15 ± 0.53 a	58.14 ± 4.53 a	72.49 ± 3.91 a	89.83 ± 5.11 a
$LSD_{0.05}$	2.52	18.81	14.77	4.47
p value	ns	ns	**	ns
ZarxZar				
well-watered	8.91 ± 0.70 a	81.03 ± 3.66 a	46.03 ± 0.52 b	87.53 ± 3.12 a
water stress	6.20 ± 0.37 b	56.58 ± 3.23 b	59.66 ± 3.44 a	90.02 ± 1.77 a
$LSD_{0.05}$	2.20	13.56	9.67	9.98
p value	*	***	*	ns
Jos ungrafted				
well-watered	10.59 ± 0.42 a	70.36 ± 1.84 a	37.91 ± 5.80 a	90.31 ± 0.18 a
water stress	5.82 ± 0.18 b	43.18 ± 1.38 b	29.19 ± 0.21 b	79.80 ± 1.44 b
$LSD_{0.05}$	1.27	6.40	6.12	4.03
p value	**	**	*	**
JosxJos				
well-watered	10.49 ± 1.08 a	58.42 ± 4.88 a	23.20 ± 0.86 a	88.56 ± 0.45 a
water stress	7.08 ± 0.04 b	41.08 ± 0.31 b	15.97 ± 4.73 b	83.68 ± 1.22 b
$LSD_{0.05}$	3.02	13.59	3.35	3.63
p value	*	*	**	*
JosxZar				
well-watered	6.41 ± 0.98 a	51.30 ± 7.64 a	26.01 ± 6.14 a	92.59 ± 4.89 a
water stress	4.94 ± 0.71 a	39.59 ± 7.12 a	20.60 ± 6.27 b	76.48 ± 3.75 b
$LSD_{0.05}$	3.36	19.02	4.39	7.14
p value	ns	ns	*	**
ZarxJos				
well-watered	10.13 ± 1.93 a	59.60 ± 8.10 a	54.39 ± 0.30 b	94.63 ± 2.12 a
water stress	7.62 ± 0.45 a	41.61 ± 3.19 a	65.54 ± 4.22 a	92.85 ± 0.01 a
$LSD_{0.05}$	4.50	24.16	10.07	5.89
p value	ns	ns	*	ns

[a] Values are the mean ± SE ($n = 9$). Means followed by the same letter in each cultivar do not differ significantly. Levels of significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

To remove precipitated protein, the assay mixture was centrifuged at 10000*g* for 15 min at 4 °C, and the absorbance of the supernatant was measured at 280 nm relative to the control.

SKDH activity was assayed in 0.1 M Tris-HCl buffer (pH 9). The reaction mixture contained 1.45 mL of 2 mM shikimic acid, 1.45 mL of 0.5 mM NADP, and 0.1 mL of supernatant. Increase of absorbance due to reduction of NADP was read over 1 min at 340 nm.[17]

PPO assay was performed in a mixture containing 2.85 mL of 50 mM potassium phosphate buffer (pH 7.0), 50 μL of 60 mM catechol, and 0.1 mL of supernatant. Increase in absorbance was read over 2 min at 390 nm.[18]

C4H activity was assayed by using the method described previously with slight modification.[19] The extract was added to 4.8 mL of reaction buffer (50 mM phosphate buffer containing 2 mM 2-mercaptoethanol, 2 mM *trans*-cinnamic acid, and 0.5 mM NADPH), which was incubated for 1 h at 37 °C. The reaction was stopped with 6 M HCl and readjusted to pH 11 with 6 M NaOH and, then, the absorbance value of the sample was measured at 280 nm.

The activity of 4CL was determined with the spectrophotometric method, using caffeic acid as the preferred phenolic substrate.[20] The reaction mixture was 5 μM *p*-coumaric acid, 50 μM ATP, 1 mM CoA-SH, and 15 mM Mg_2SO_4. The reaction mixture was incubated at 40 °C for 10 min, and then the absorbance was measured at 333 nm.

GPX activity was determined by following the previously reported method.[21]

The protein concentration of the extracts was determined according to the method of Bradford,[22] using bovine serum albumin as the standard.

Statistical Analysis. Data compiled were submitted to an analysis of variance (ANOVA), and differences between the means were compared by Duncan's multiple-range test ($p > 0.05$).

RESULTS

RGR and LRWC. The total biomass diminished significantly in cv. Josefina as well as in *JosxJos* under water-stress conditions. Cv. Zarina did not appear to be affected by the stress, whereas its self-graft had a negative effect on total biomass, with a reduction of 41% with respect to control. In terms of reciprocal grafts, none presented a reduction of total biomass under water-stress conditions, values being higher in the graft *ZarxJos* in both treatments, well-watered and water stress (Table 1).

In cv. Josefina and its self-graft, the total RGR declined under stress conditions. In contrast, cv. Zarina was not significantly affected, although a 30% loss was found for *ZarxZar* with respect to the well-watered treatment. In the grafts *JosxZar* and *ZarxJos*,

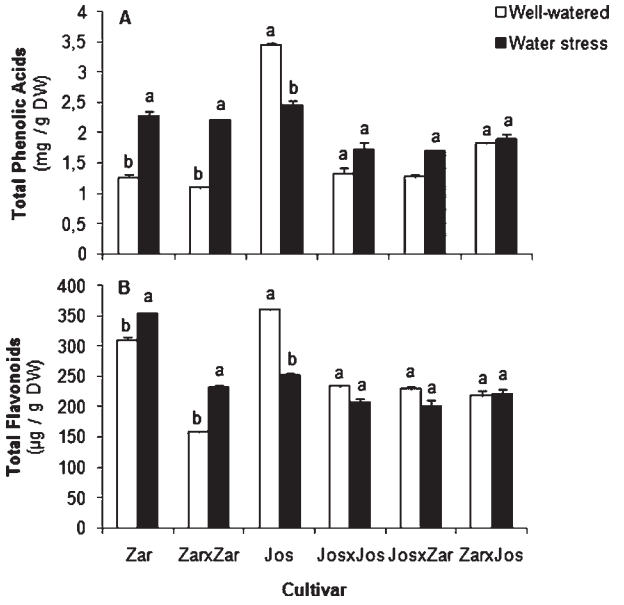

Figure 1. Influence of moderate water stress on phenolic acids and flavonoids total in ungrafted, grafted, and self-grafted tomato plants. Columns represent the mean ± SE (n = 9). Means accompanied by the same letter in each cultivar do not differ significantly.

no differences were observed under stress conditions. In terms of biomass, the graft *ZarxJos* maintained the highest levels of total RGR (Table 1).

In cv. Josefina as well as *JosxJos*, the root RGR fell with the water-deficit treatment. Cv. Zarina increased its RGR to 88% under stress conditions, whereas its self-graft was not visibly affected. The reciprocal grafts behaved in the contrary way, with root RGR in *JosxZar* decreasing 21% under stress and RGR for *ZarxJos* increasing 20% (Table 1).

LRWC is a reliable indicator of the capacity of the plant to re-establish its water balance after a water-deficit situation, as well as the capacity to tolerate this stress. Only cv. Josefina, its self-graft, and *JosxZar* showed a significant decline in LRWC with the stress applied, whereas the rest were not appreciably affected (Table 1).

Phenolic Compounds. The phenolic qualities and quantities of tomato upon environmental stress and modulated by the genetic variability are of great interest from the point of view of the tomato plant health and the quality of the fresh produce because tomato is one of the most important vegetables worldwide.[23] The total content in phenolic acids increased significantly under water-stress conditions in cv. Zarina, its self-graft, and *JosxZar*, whereas in cv. Josefina, a decline of 30% was registered with respect to its control (Figure 1A). The total flavonoids increased in concentration only in cv. Zarina and *ZarxZar*, whereas cv. Josefina showed a decrease under stress conditions (Figure 1B).

The hydromethanolic extracts of tomato leaves showed major peaks of phenolic metabolites that have been previosuly reported in the literature and were characterized by HPLC-PDA-ESI-MS/MS (negative ionization mode)[24] as presented in the Figure 2, besides other compounds present in small amounts. Two caffeoylquinic acids were characterized (compounds **1** and **2**) (Figure 2) by means of $-MS^2$ of their deprotonated molecular ion (m/z 353), giving a base peak at m/z 191. In compound **1** a relatively intense ion at m/z 179 is also observed, whereas in **2** this ion is weak or undetectable and, according to a previous

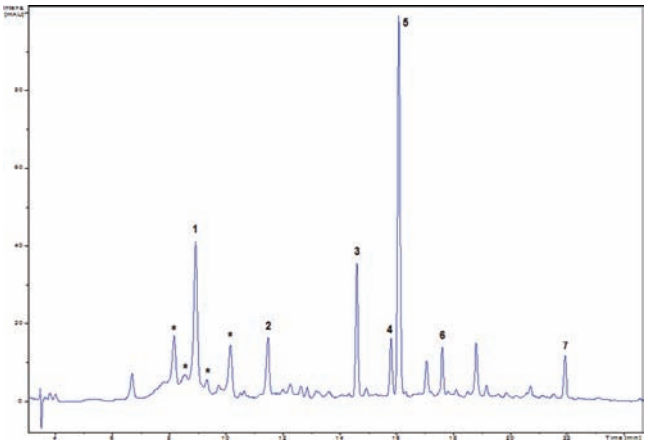

Figure 2. HPLC chromatogram (280 ± 10 nm) of extract of leaf of tomato revealing the presence of seven peaks: (1) 3-caffeoylquinic acid; (2) 5-caffeoylquinic acid; (3) quercetin 3-apiosyl rutinoside; (4) kaempferol 3-apiosyl rutinoside; (5) quercetin 3-rutinoside (rutin); (6) kaempferol 3-rutinoside; (7) dicaffeoylquinic acid; (∗) CQA derivatives.

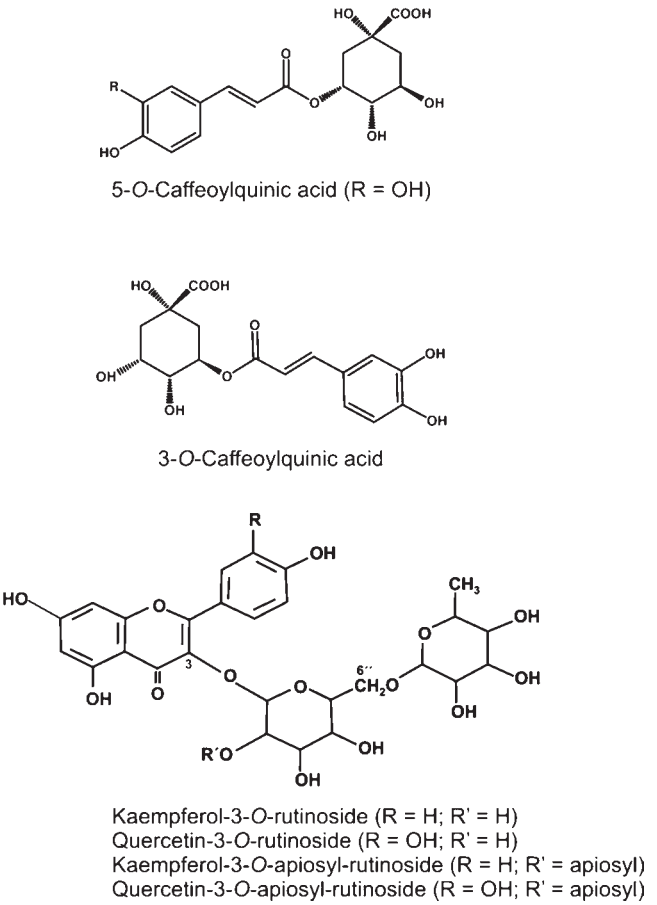

Figure 3. Structures of phenolic acids and flavonoids (**1**−**7**).

paper,[25] can be labeled as 3-caffeoylquinic acid (**1**) and 5-caffeoylquinic acid (**2**), respectively. Additionally, we also detected a dicaffeoylquinic acid ($[M - H]^-$, m/z 515) (**7**) (Figure 3).

With respect to the flavonoids, in the UV chromatogram of the tomato leaf hydroalcoholic extracts (Figure 2) we have detected

Table 2. Quantities of Phenolic Compounds in Ungrafted, Grafted, and Self-Grafted Tomato Plants[a]

	compound[b]							
	hydroxycinnamic acids and derivatives				flavonoids and glycosides			
cultivar/water treatment	3-CQA (μg/g DW)	5-CQA (μg/g DW)	di-CQA (μg/g DW)	CQA derivatives (mg/g DW)	Querc-3-api-rut (μg/g DW)	Querc-3-rut (μg/g DW)	Kaemp-3-api-rut (μg/g DW)	Kaemp-3-rut (μg/g DW)
Zar ungrafted								
well-watered	172.0 ± 11.5 b	326.2 ± 27.5 a	156.8 ± 10.1 b	0.61 ± 0.09 b	41.6 ± 3.6 b	234.6 ± 13.2 a	16.4 ± 1.9 b	17.4 ± 1.7 a
water stress	251.7 ± 5.7 a	383.0 ± 17.3 a	436.0 ± 26.6 a	1.20 ± 0.11 a	61.6 ± 3.9 a	241.3 ± 9.0 a	32.0 ± 1.4 a	17.8 ± 0.9 a
$LSD_{0.05}$	35.9	90.2	79.1	0.41	14.9	44.58	6.6	5.5
p value	**	ns	***	*	*	ns	**	ns
ZarxZar								
well-watered	188.5 ± 17.1 b	93.0 ± 7.4 b	189.3 ± 10.0 b	0.63 ± 0.06 b	29.8 ± 0.4 b	115.3 ± 1.9 b	8.5 ± 0.2 b	5.1 ± 0.1 b
water stress	330.7 ± 14.8 a	244.6 ± 9.8 a	341.9 ± 6.0 a	1.30 ± 0.05 a	41.7 ± 1.7 a	165.9 ± 4.9 a	13.4 ± 0.6 a	9.8 ± 0.4 a
$LSD_{0.05}$	62.9	34.1	32.5	0.23	5.0	14.7	1.7	1.1
p value	***	***	**	*	*	*	*	**
Jos ungrafted								
well-watered	525.7 ± 12.5 a	494.2 ± 2.9 a	447.6 ± 12.8 a	1.99 ± 0.04 a	42.2 ± 1.1 a	189.1 ± 2.7 a	12.9 ± 0.4 a	11.2 ± 3.8 a
water stress	447.5 ± 12.3 b	258.4 ± 1.4 b	331.7 ± 46.3 b	1.42 ± 0.01 b	53.4 ± 1.7 a	172.1 ± 2.7 b	16.4 ± 1.4 a	10.8 ± 0.1 a
$LSD_{0.05}$	48.8	9.1	77.5	0.12	15.6	12.7	4.3	13.6
p value	*	***	*	*	ns	**	ns	ns
JosxJos								
well-watered	192.1 ± 5.1 a	154.1 ± 23.3 a	248.5 ± 5.5 b	0.89 ± 0.09 a	36.3 ± 0.3 a	171.5 ± 1.4 a	14.3 ± 0.1 a	11.2 ± 0.2 a
water stress	172.5 ± 5.9 a	84.8 ± 2.7 b	735.0 ± 13.2 a	0.74 ± 0.01 a	36.2 ± 0.9 a	144.3 ± 2.0 b	14.8 ± 0.5 a	10.9 ± 0.4 a
$LSD_{0.05}$	21.9	65.3	39.8	0.26	2.8	6.9	1.5	1.3
p value	ns	**	***	ns	ns	*	ns	ns
JosxZar								
well-watered	226.3 ± 9.1 b	126.9 ± 3.6 a	198.5 ± 23.3 b	0.82 ± 0.05 b	34.2 ± 1.7 a	170.4 ± 4.4 a	13.1 ± 0.4 a	12.1 ± 0.7 a
water stress	262.3 ± 7.9 a	142.1 ± 5.1 a	292.2 ± 9.2 a	1.02 ± 0.03 a	36.2 ± 0.3 a	159.0 ± 4.2 a	14.6 ± 0.8 a	11.9 ± 0.2 a
$LSD_{0.05}$	33.5	17.5	74.7	0.18	4.9	16.9	2.5	2.3
p value	*	ns	**	*	ns	ns	ns	ns
ZarxJos								
well-watered	317.1 ± 9.5 a	225.5 ± 4.3 a	210.4 ± 2.1 b	1.08 ± 0.03 a	47.6 ± 1.1 a	156.1 ± 1.8 a	14.7 ± 0.8 a	10.2 ± 0.2 a
water stress	284.8 ± 17.2 a	201.0 ± 11.2 a	348.1 ± 15.5 a	1.08 ± 0.06 a	41.6 ± 2.5 a	138.0 ± 7.6 a	12.2 ± 0.3 a	9.6 ± 0.7 a
$LSD_{0.05}$	54.8	33.3	43.4	0.20	7.7	21.9	2.6	2.1
p value	ns	ns	**	ns	ns	ns	ns	ns

[a] Values are the mean ± SE ($n = 9$). Means followed by the same letter in each cultivar do not differ significantly. Levels of significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. [b] CQA, caffeoylquinic acid; Querc, quercetin; Kaemp, kaempferol; rut, rutinoside; api, apiosyl.

four compounds (**3**−**6**). Compound **5** present in all samples showed UV spectra of quercetin derivative[25] and deprotonated molecular ion at *m/z* 609. Its MS^2 fragmentation gave the unique peak at *m/z* 301 corresponding to the deprotonated ion of its aglycone, as well as an absence of intermediate ions, indicating a interglycosidic linkage rhamnosyl (1→6) glucoside (rutinoside).[26] All of these data indicate that this compound is quercetin-3-*O*-rutinoside (rutin), widely distributed in tomato cultivars[23] (Figure 3).

The other quercetin derivative (**3**) present in the samples showed in −MS a deprotonated molecular ion 132 Da more intense than in **5** (*m/z* 741), which could indicate that the compound is a pentosyl derivative of **5**. The MS^2 fragmentation of this compound is similar to that observed for quercetin-3-*O*-(2″-pentosyl-6″-rhamnosyl) glucoside characterized in tomato,[27] of which the structure was also described in tomato[23] as quercetin-3-*O*-(2″-apiosyl-6″-rhamnosyl) glucoside (quercetin-3-*O*-(2″-apiosyl) rutinoside) (Figure 3).

Compounds **4** and **6** show UV spectra of kaempferol derivatives,[25] and their deprotonated molecular ions are 16 amu lower than in **3** and **5**, respectively (*m/z* 725 and 593). In this sense, their MS fragmentations are similar to those of **3** and **5** and differentiated only in the ion of the deprotonated aglycone, and it would be tentatively labeled as kaempferol-3-*O*-(6″-rhamnosyl) glucoside (kaempferol-3-*O*-rutinoside) (**6**) identified in different works on tomato[28,29] and the kaempferol-3-*O*-(2″-apiosyl-6″-rhamnosyl)glucoside(kaempferol-3-*O*-(2″-apiosyl)rutinoside) (**4**) by comparison with the previously described data for the quercetin derivatives. Recent studies[23,28] described in tomato a kaempferol rutinoside pentoside that should coincide with our compound **4**.

The quantities were determined for the derivates of caffeolylquinic acid and flavonols (quercetin and kaempferol glycosyl derivatives) present in the leaves to determine the possible alteration of the levels of these metabolites as a consequence of

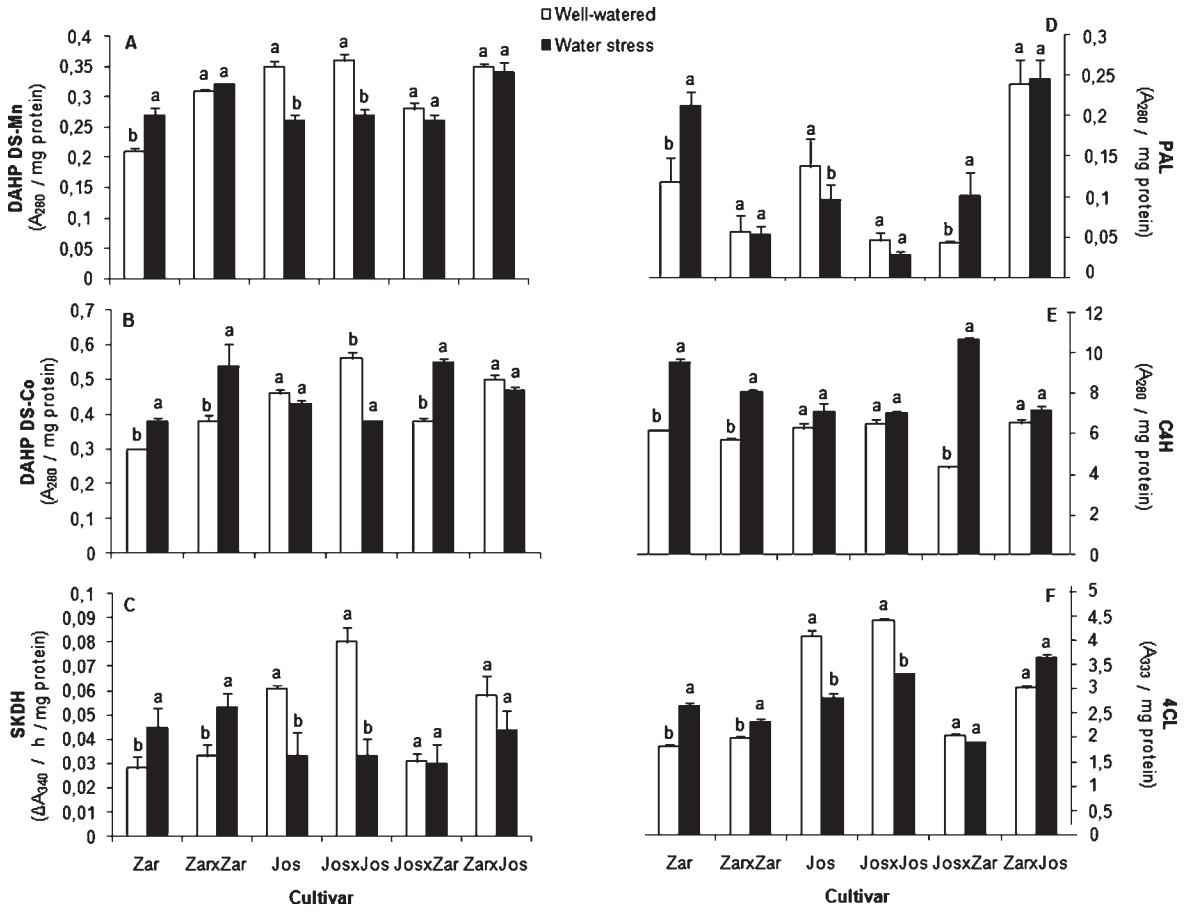

Figure 4. Influence of moderate water stress on flavonoid and phenylpropanoid synthesis-related enzyme activity in ungrafted, grafted, and self-grafted tomato plants. Columns represent the mean ± SE (n = 9). Means accompanied by the same letter in each cultivar do not differ significantly.

the water stress applied. Cv. Zarina as well as *ZarxZar* registered a significant increase under water-deficit conditions in practically all of the hydroxycinnamic acids and derivatives found (Table 2). In contrast, in cv. Josefina under water-stress conditions these metabolites diminished with respect to their control (Table 2). In *JosxJos* the quantity of 5-caffeoylquinic acid declined, whereas the quantity of dicaffeoylquinic acid increased (Table 2). The graft *JosxZar* showed a general increase in the compounds studied, whereas in *ZarxJos* only the quantity of dicaffeoylquinic acid increased under stress (Table 2). With respect to flavonoids and derivatives, cv. Zarina registered a significant rise in the compounds quercetin 3-apiosyl rutinoside and kaempferol 3-apiosyl rutinoside under the stress conditions applied (Table 2). However, its self-graft showed an increase in all of the flavonoids found. Both cv. Josefina and its self-graft, under water stress, showed reductions in the quantities of quercetin 3-rutinoside and kaempferol 3-rutinoside with respect to their control (Table 2). In the reciprocal grafts, no changes were found in the quantities of flavonoids and derivatives with water stress (Table 2).

Activities of Flavonoid and Phenylpropanoid Synthesis and Degradation-Related Enzymes. To study the synthesis of phenolic compounds, we analyzed the activity of the main enzymes in the shikimate pathway. The activity of the enzymes DAHP (DS-Mn, DS-Co, EC 4.1.2.15), SKDH (EC 1.1.1.25), PAL (EC 4.3.1.5), C4H (EC 1.14.13.11), and 4CL (EC 6.2.1.12) significantly increased in cv. Zarina, as well as in its self-graft with respect to its control, except for DAHP-DS Mn activity in cv. Zarina self-grafted plants (Figure 4). In contrast, both the cv. Josefina and its self-graft diminished in the activity of these enzymes under moderate water stress, except for C4H activity (Figure 4). The graft *JosxZar* increased only in the activity of the enzymes DAHP DS-Co, PAL, and C4H with respect to control, whereas the rest of the enzymes studied did not register changes in activity under stress (Figure 4). Finally, *ZarxJos* showed no alterations in activity in any of the enzymes of the cycle under the treatment applied (Figure 4).

In terms of the enzymes in charge of the degradation of phenols, polyphenol oxidase (PPO, EC 1.10.3.2) and guaicol peroxidase (GPX, EC 1.11.1.7), cv. Zarina showed a decline in the activity of both enzymes (Figure 5), whereas PPO activity fell only in *ZarxZar* under water-stress conditions (Figure 5). Only the graft *JosxJos* presented an increase in the activity of these enzymes with respect to its control (Figure 5). However, activity in cv. Josefina and the reciprocal grafts was not affected by the stress (Figure 5).

DISCUSSION

Environmental stress exerts the most limiting conditions for horticultural productivity. Thus, it has been amply demonstrated that water stress can limit growth,[30] and thus the capacity of plants to tolerate this stress is of crucial economic importance. A current method of adapting plants to environmental stress is grafting.[31] Our results show neither the total biomass nor the

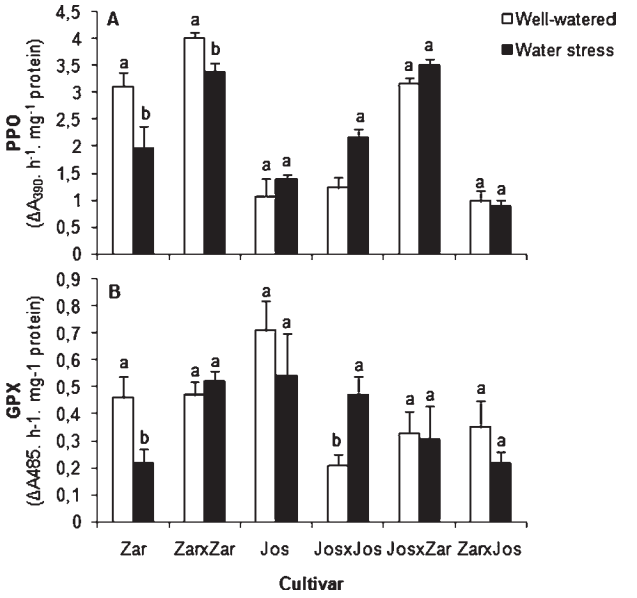

Figure 5. Influence of moderate water stress on flavonoid and phenylpropanoid degradation-related enzymes activity in ungrafted, grafted, and self-grafted tomato plants. Columns represent the mean ± SE (n = 9). Means accompanied by the same letter in each cultivar do not differ significantly.

total RGR was affected with cv. Zarina, whereas cv. Josefina declined in growth under stress conditions (Table 1). These results coincide with those found in our previous work.[12] The grafts *ZarxZar* and *JosxJos* showed decreases in these parameters under stress conditions; however, the total biomass was greater in the self-grafts with respect to the cultivars Zarina and Josefina (Table 1). These results are consistent with a recent paper,[32] in which it was observed that the graft itself had a positive effect on growth, due possibly to a hormonal effect. Growth in the grafts*JosxZar* and *ZarxJos* was not appreciably affected by stress (Table 1).

LRWC is considered to be a reliable indicator of the plant's capacity to return to a favorable state after water deficit and also of its capacity to tolerate this stress.[12] Cv. Zarina, its self-graft, and *ZarxJos* maintained the LRWC under stress conditions, which could be associated with greater growth of the root system (Table 1). Thus, the graft *ZarxJos* registered an increase in the root RGR under stress conditions, this possibly being associated with better LRWC. These results confirm, on the one hand, that the use of the tolerant cultivar (Zarina) as rootstock transmits resistance to the water-stress-sensitive shoot as in the case of cv. Josefina. Also, the graft *JosxZar* did not lose biomass or total RGR under stress conditions, but it proved less effective than *ZarxJos*, as a decline in LRWC was noted, possibly due to the low root RGR.

In response to water stress, plants can accumulate a broad range of antioxidants, including phenolic compounds.[33] Our results show that in cv. Zarina, its self-graft, and *JosxZar* the concentration of phenolic compounds increased after water stress (Table 2). This could be correlated with the greater activity of synthesis enzymes (Figure 4) as well as with a decrease in phenol-degrading enzymes (Figure 5). Prior studies have demonstrated that an increase in the phenolic concentration, as well as in the enzyme PAL, could be correlated with better drought resistance.[34,35] In this case, the shoot of Zarina had a stronger influence on phenolic metabolism than did the rootstock. In turn, a greater phenolic content was related to improved growth in grafted plants.[32,36] These coincide with our results.

The individual analysis of the phenolic compounds indicated that the cv. Zarina and its self-graft increased the concentration of most of the hydroxycinnamic acids and derivatives, this increase being more pronounced in dicaffeoylquinic acid . Also, the content in the flavonoids studied was found to be higher (Table 2). In contrast, cv. Josefina and its self-graft showed a contrary trend, with a significant decline in most of these compounds under water stress (Table 2). In both self-grafts, the quantity of each compound identified was relatively lower than that registered for its respective ungrafted cultivar. These results coincide with those recently reported in watermelon plants,[8] because we found that the grafted cultivars maintained lower concentrations of kaempfeol than did the cultivars used as ungrafted rootstocks. In the reciprocal grafts, only *JosxZar* increased in hydroxycinnamic acids studied under water stress (Table 2). This appears to indicate that the use of cv. Zarina as a shoot is important in providing a greater phenolic content in the leaf, whereas its use as a rootstock did not substantially improve the phenolic metabolism or the accumulation of phenolic metabolites.

Our results are important from the standpoint of improving water-stress resistance in the cultivation of cherry tomato by the use of grafting. The self-grafts displayed a behavior very similar to that of the ungrafted cultivars, but it was the graft itself that prompted the fall in phenolic content. This could be explained by low transfer efficiency of phenolic compounds from the rootstock to the scion. In addition, the use of the water-stress-tolerant cv. Zarina as a rootstock improves the growth of cv. Josefina under moderate water deficit. This may be due to better root RGR and the maintenance of the LRWC, although other processes could be involved, and more studies are needed to clarify this point. Besides, the use of cv. Zarina as a shoot for grafted plants maintains growth under stress conditions, which might be explained by the higher phenolic metabolism, although other processes could also be involved. However, this graft reflects a certain stress with the decline in LRWC under water stress, due possibly to the decrease in root RGR. These results could indicate that phenolic metabolism is more influenced by the aerial part, and therefore we conclude that the capacity of inducing tolerance in rootstocks depends on the genotype of the shoot.

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ABBREVIATIONS USED

4CL, 4-coumarate coenzyme A ligase; C4H, cinnamate 4-hydroxylase; DAHPS, 3-deoxy-7-phosphoheptulonate synthase; GPX, guaiacol peroxidase; LRWC, leaf relative water content; PAL, phenylalanine ammonia-lyase; PPO, polyphenol oxidase; SKDH, shikimate dehydrogenase.

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