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Variations of leaf morphology, photosynthetic traits and water-use efficiency in Western-Mediterranean tomato landraces

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Abstract

Modern tomato (*Solanum lycopersicum* L.) breeding has mainly focused on increasing productivity under unlimited watering. In contrast, some Mediterranean accessions have been traditionally cultivated under water shortage and selected on the basis of their water-use efficiency (WUE). Ramellet and Penjar landraces were planted with other traditional, old and modern inbreeds, under full irrigation. In order to found differences between the tomato accessions, gas-exchange and leaf morphology measurements were performed. Despite high variability, Ramellet and Penjar presented clear differences compared to modern cultivars, mostly related to leaf morphology and photosynthetic traits, while no differences were found in WUE. Results highlighted that better leaf CO_2 conductance might be a main factor determining the improvement of net CO_2 assimilation and WUE.

Additional key words: carbon assimilation; carbon isotope composition; diffusive limitations; mesophyll conductance.

Introduction

Tomato (*Solanum lycopersicum* L.) is the second most produced and consumed vegetable crop in the world and it is an integral part of the human diet (FAO 2015). The tomato crop is widely distributed, adapted to a wide variety of climates (Cuartero and Fernández-Muñoz 1998), with a large number of landraces, cultivars, and mutants, and infertile with many wild relative species. Consequently, it has become a plant model for fleshy fruit studies (The Tomato Genome Consortium 2012).

Tomato wild relatives have been a major source for improving traits of the domesticated tomato, mostly regarding to biotic stress resistances (Bai and Lindhout 2007, Foolad and Panthee 2012, Xu *et al.* 2013), while abiotic stress resistance has largely been neglected (Foolad 2007). Unlike biotic resistances, tolerance to abiotic stress generally presents a polygenic control and thus, the use of wild relatives as a gene source frequently leads to problems related to linkage drag. Alternatively, tomato

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Abbreviations: AG – accession groups; BRA – Balearic Islands Ramellet accessions; C_a – CO_2 ambient concentration; C_c – chloroplastic CO_2 concentration; Chl – chlorophyll; C_i – substomatal CO_2 concentration; CON – control accessions; CONm – modern inbreeds accessions; CONo – old varieties accessions; CONt – traditional non-long shelf-life fruit genotype accessions; CPA – Catalonian Penjar accessions; ETR – electron transport rate; g_m – mesophyll conductance; g_s – stomatal conductance to H_2O; g_{total} – the total conductance; LD – leaf density; $\delta^{13}C$ – leaf isotope composition; LMA – leaf mass area; LSL – long shelf-life fruit phenotype; LT – leaf thickness; PCA – principal components analyses; P_N – net photosynthetic rate; R_D – rate of mitochondrial respiration at darkness; R_L – rate of mitochondrial respiration in light; V_{cmax} – maximum velocity of Rubisco carboxylation; VPA – Valencian Country Penjar accessions; WM – Western Mediterranean accessions; WUE – water-use efficiency; WUE_i – intrinsic water-use efficiency.
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landraces represent an important source of genes readily available for tomato breeding likely reducing the time needed for introgression of the tolerance gene and at the same time decreasing the possibilities of linkage drag. In fact, unlike modern cultivars, landraces have been selected to produce under particular/local conditions, which could even represent rash climate condition, and therefore it could be an important source for abiotic stress-adaptive traits.

In future, open field-grown tomatoes will be exposed to the effect of climate change (Gitay *et al.* 2002, Sheffield and Wood 2008, Gornall *et al.* 2010). In this scenario of higher water demand in some Southern European countries, the increase of the crop WUE is a primary issue (Condon *et al.* 2004, Gago *et al.* 2014). WUE represents the capacity of a crop to produce biomass per unit of water consumed. It can be estimated as the ratio of carbon gain to water consumption at different levels and time scales (Donovan and Ehleringer 1994, Flanagan and Farquhar 2014, Gago *et al.* 2014). Thus, at the leaf level, intrinsic WUE (WUE_i) can be estimated using instantaneous gas-exchange measurements, relating the net photosynthetic rate (P_N) to the stomatal conductance to H_2O (g_s). Most abiotic stresses reduce plant growth through stomatal limitations (*i.e.* stomatal closure – g_s reduction), which unavoidably leads to limited CO_2 intake (P_N reduction) (Condon *et al.* 2002, Medrano *et al.* 2002, Hetherington and Woodward 2003). Thus, WUE_i improvement can be achieved at the biochemical (*e.g*. Long *et al.* 2006) and/or at morphological level (*e.g.* Flexas *et al.* 2008).

Alternatively, WUE can be estimated by measuring the isotope ^{13}C composition in leaf tissues, which supplies information integrated over longer time. Both methods have long been recognized as reliable to measure plant WUE (*e.g.* Farquhar *et al.* 1982, Martin and Thorstenson 1988, Donovan and Ehleringer 1994, Soolanayakanahally *et al.* 2009, Galmés *et al.* 2011, Dhanapal *et al.* 2015), although taken carefully (Seibt *et al.* 2008).

In this regard, the Mediterranean region, considered a secondary center of origin for tomato, appears to be a repository of tomato landraces adapted to produce in open field under hot and dry Mediterranean summer conditions (*e.g.* Bota *et al.* 2014). Particularly, adaptation leading to improved WUE_i have already been described in Ramellet tomato as compared to commercial tomato varieties (Galmés *et al.* 2011, 2013). Ramellet tomato represents a population of landraces from the Balearic Islands, diverse in terms of plant and fruits traits, but all exhibiting the long shelf-life fruit phenotype (LSL) (Ochogavia *et al.* 2011, Bota *et al.* 2014). This fruit phenotype relates Ramellet tomato to other Western Mediterranean landraces such as Penjar tomato from the Valencian Country and Catalonia, which have also been selected under similar criteria and environmental conditions as Ramellet (Casals *et al.* 2011).

In this study, screening of leaf morphology, photosynthetic traits, and WUE was performed on a selection of Ramellet and Penjar tomato accessions (*i.e.* Western Mediterranean accessions, WM), and compared to a diverse array of control accessions (CON), including traditional non-LSL tomatoes (CONt), old varieties (CONo), and modern tomato inbreeds (CONm). The hypothesis of this experiment surmised that growth conditions of WM accessions would increase photosynthesis capacity and WUE.

Particularly, the objectives of the study were: (*1*) to study the variability in leaf morphological and photosynthetic traits between the WM and CONt, CONo or CONm accessions, and (*2*) within the WM accessions, to correlate variation with the geographic origin, and (*3*) to search for the underlying determinants of the variation in the photosynthetic capacity and the CO_2 diffusive parameters and their relation with WUE. Results may reveal important traits which can be used in future tomato breeding and also show if there is any degree of variation of such traits within Ramellet and Penjar landraces. Many of the adaptive traits in WM landraces could be of interest under the expected climate change scenario.

Materials and methods

Plant material and experimental conditions: For this study, 31 accessions of tomato (*S. lycopersicum* L.) were selected. Two major groups were differentiated, Penjar and Ramellet of Eastern Iberian peninsula and Balearic Islands landraces, bearing the LSL-fruit phenotype and selected under poor irrigation conditions (*i.e.* WM, 21 accessions), and control accessions (CON, 10 accessions). The accessions of both WM and CON were subdivided into different accession groups (AG). WM were separated in three AGs according to their geographic origin, thus Ramellet tomatoes from the Balearic Islands (BRA, seven accessions), Penjar tomatoes from Catalonia (CPA, nine accessions), and Valencian Country (VPA, five accessions). In turn, CON were also divided into three AGs, modern inbreeds (CONm, three accessions), old varieties selected *ca.* 1950 (CONo, three accessions), and traditional, nonLSL varieties (CONt, four accessions). Seeds of all accessions were obtained from the University of Balearic Islands (UIB), Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV), Fundació Miquel Agustí, Tuscia University of Viterbo and S. Soler collection seed banks (*see* the text table below).

The experiment was performed in Alcossebre (Valencian Country, Spain), at a commercial field used to grow Penjar tomato by the association of tomato producers (Tomata de Penjar d'Alcalà de Xivert). Seedlings were grown in trays of 104 wells prior field transplantation. Crop management was performed using standard practices

List of the accessions studied in this experiment. From left to right, columns indicate the accession code, the accession group (AG) (CON refers to the control, BRA refers to Balearic, CPA to Catalonian and VPA to Valencian Country origin), the local name, the geographic origin and the seed bank code. [1]University of the Balearic Islands (UIB), [2] Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV), [3]Fundació Miquel Agustí, [4] Tuscia University of Viterbo, [5]S. Soler collection seed banks.

Accession code	AG	Local name	Geographic origin	SB code
CONm 1	CON	DZ52	Modern cultivar	UPV37326 [2]
CONm 2	CON	9457 cherry	Modern cultivar	UPV37327 [2]
CONm 3	CON	9466 jj	Modern cultivar	UPV37328 [2]
CONo 1	CON	Monalbo	Modern cultivar	V710150 [4]
CONo 2	CON	Ailsa Craig	Modern cultivar	V710171 [4]
CONo 3	CON	M82	Modern cultivar	V711278 [4]
CONt 1	CON	20 SMEC-3	Pomigliano d'Arco, Naples (Italy)	UPV37323 [2]
CONt 2	CON	Makedonia	Thessaloniki (Greece)	UPV37306 [2]
CONt 3	CON	Santorini	Thessaloniki (Greece)	UPV37307 [2]
CONt 4	CON	Pera Abruzzo	Terrano, Abruzzo (Italy)	V710314 [4]
BRA 1	BRA	Ramellet	Felanitx, Balearic Islands (Spain)	UIB0015 [1]
BRA 2	BRA	Ramellet	Llucmajor, Balearic Islands (Spain)	UIB0018 [1]
BRA 3	BRA	Ramellet	Sóller, Balearic Islands (Spain)	UIB0019 [1]
BRA 4	BRA	Ramellet branca llarga	Banyalbufar, Balearic Islands (Spain)	UIB0031 [1]
BRA 5	BRA	Penjar	Santanyí, Balearic Islands (Spain)	BGV001573 [2]
BRA 6	BRA	Ramellet	Santa Margalida, Balearic Islands (Spain)	UIB0098 [1]
BRA 7	BRA	Ramellet	Balearic Islands (Spain)	UIB0118 [1]
CPA 1	CPA	LC 95 Montserrat	Catalonia (Spain)	LC95 [3]
CPA 2	CPA	Penjar (punxa) Barratxina	Sabadell, Catalonia (Spain)	LC126 [3]
CPA 3	CPA	Poma	Torroella de Montgrí, Catalonia (Spain)	BGV002089 [2]
CPA 4	CPA	Penjar	Mollet de Vallès, Catalonia (Spain)	BGV002192 [2]
CPA 5	CPA	Petit	Vendrell, Catalonia (Spain)	BGV002243 [2]
CPA 6	CPA	Penjar	Arenys de Munt, Catalonia (Spain)	LC360 [3]
CPA 7	CPA	Penjar	Lleida, Catalonia (Spain)	LC369 [3]
CPA 8	CPA	Tomacó de fulla grossa	El Catllar, Catalonia (Spain)	FMA171 [3]
CPA 9	CPA	Penjar	Les Piles, Catalonia (Spain)	FM31 [3]
VPA 1	VPA	Penjar	Alcalà de Xivert, Valencian Country (Spain)	ALX1 [5]
VPA 2	VPA	Gran	Castelló, Valencian Country (Spain)	BGV005509 [2]
VPA 3	VPA	Estrella	Alcalà de Xivert, Valencian Country (Spain)	ADX1 [5]
VPA 4	VPA	Punteta	Alcalà de Xivert, Valencian Country (Spain)	ADX2 [5]
VPA 5	VPA	Moradeta	Alcalà de Xivert, Valencian Country (Spain)	ADX3 [5]

for the cultivation of Penjar tomato. Chicken manure (8,500 kg ha^{-1}) was applied as basal fertilizer before plantlet transplantation. Three-months-old seedlings from all accessions were transplanted to the field. For each accession, at least four plants were planted and separated by 0.5 m. Plants were irrigated every two days by a black plastic-covered dripping system to ensure homogeneous water availability for all plants. Weeds were removed manually. Plants were trained with bamboo canes and left without pruning after the first inflorescence.

All measurements and samplings were performed on the youngest fully expanded leaves. Environmental conditions during the experiment were those typical of the Mediterranean summer, with high temperatures and low relative humidity. The average temperature during May-August was 23.3ºC and the mean monthly evapotranspiration was 149.9 L m^{-2}.

Leaf gas exchange and chlorophyll (Chl) *a* fluorescence were measured simultaneously with an open infrared gas-exchange analyzer equipped with a leaf chamber fluorometer (*Li-6400-40*, *Li-Cor Inc*., Lincoln, USA). Measurements were performed from 9:00 to 19:00 h. Preliminary tests confirmed insignificant differences between early morning and late afternoon measurements (data not shown).

Environmental conditions in the leaf chamber and measurements were done according to Galmés *et al.* (2011). The leaf temperature was between 31–33ºC (corresponding to the average daily leaf temperature during the period of measurement), a vapor pressure deficit of 1.74 ± 0.27 kPa, an air flow of 500 µmol(air) min^{-1}, and CO_2 ambient concentration (C_a) measurements for the curve were set at 400, 0, 100, 200, 300, and 2,000 µmol(CO_2) mol^{-1}(air). Four to five curves per an accession were performed on different plants. Corrections for CO_2 leakage in and out of the leaf chamber of the *Li-6400-40* were applied to all gas-exchange data, as described by Flexas *et al.* (2007).

The parameters related to the quantum efficiency of the PSII-driven electron transport and the rate of electron transport (ETR) and the rate of mitochondrial respiration

at darkness (R_D) were calculated according to Galmés *et al.* (2011).

The mesophyll conductance to CO_2 (g_m) was estimated according to Harley *et al.* (1992):

$$g_m = P_N / C_i - \{\Gamma^*[ETR + 8(P_N + R_L)]/[ETR - 4(P_N + R_L)]\},$$

where the rate of mitochondrial respiration in light (R_L) was calculated as the half of the R_D. The chloroplast CO_2 compensation point (Γ^*) was calculated using the *in vitro* Rubisco specificity factor ($S_{c/o}$) of *S. lycopersicum* from Hermida-Carrera *et al.* (2016) and the oxygen concentration (210,000 µmol mol^{-1}) as in Galmés *et al.* (2011).

P_N-C_i curves were transformed into P_N *vs.* chloroplastic CO_2 concentration (C_c) curves using estimated values of g_m. From P_N-C_c curves, the maximum velocity of Rubisco carboxylation (V_{cmax}) was calculated as by Bernacchi *et al.* (2002), but using *S. lycopersicum* specific values of Rubisco kinetics adjusted to the measured leaf temperature (Hermida-Carrera *et al.* 2016).

Leaf morphology parameters: Leaf thickness (LT) was measured using a digital leaf caliper in the middle part of the terminal leaflet, from the edge to the center, avoiding major leaf veins. Measurements (n = 4) were taken in different plants for each accession.

The same leaves were used to obtain the leaf mass per area (LMA), calculated as the ratio of dry mass to leaf area. All leaflets were considered but excluding the leaf rachis. Leaf area was determined from images of leaflets using an image analyzer program (*ImageJ 1.49v*, *National Institutes of Health*, USA). Dry mass was determined from oven drying leaflets at 70ºC until constant mass (*ca*. 72 h). Leaf density (LD) was calculated from LMA and LT.

Leaf $\delta^{13}C$ isotope composition: Leaves used to calculate the leaf morphology parameters were also used to determine the carbon isotopic composition. Dry leaf samples were ground to fine powder and were sampled for isotope analysis.

Samples were combusted in an elemental analyzer (*Thermo Flash EA 1112 Series*, Bremen, Germany), and CO_2 was directly injected into a continuous-flow isotope ratio mass spectrometer (*Thermo-Finnigan Delta XP*, Bremen, Germany) for isotope analysis. Peach leaf standards (NIST 1547) were run every six samples. The standard deviation of the analysis was below 0.1‰. Results are presented as δ *vs.* PDB.

Statistical analysis: Two nested analysis of variance (nested *ANOVA*) were performed, with the accessions nested within their respective geographical origin, using the following parameters: P_N, R_D, g_s, g_m, the total conductance (g_{total}) as:

$$g_{total} = 1/[(1/g_s) + (1/g_m)]$$

and C_c, V_{cmax}, ETR, WUE_i, g_m/g_s, leaf $\delta^{13}C$, LMA, LT, and LD. The first nested *ANOVA* was performed to check for differences between the WM and CON accessions, and the second nested *ANOVA* to search for differences between BRA, CPA, VPA, and CON accession groups. In both nested *ANOVA*s, significant differences between means were revealed by *Duncan'*s analyses (P<0.05).

A principal components analysis (PCA) was performed, including all 32 accessions together, using the same parameters as in the nested *ANOVA* but excluding LD and g_m/g_s. A cluster analysis was performed for only WM accessions using the same parameters as in the PCA analyses. *Pearson*'s correlations (R) were calculated to determine the relationships among the studied parameters. All statistical analyses were performed using *R software* (*ver. 3.2.2*; *R Core Team*, Vienna, Austria).

Results

Large variability of morphological and photosynthetic parameters in the main leaf of tomato accessions: The studied accessions showed a notable variation in the leaf morphological parameters LT, LD, and LMA (Table 1).

LT varied from 0.30 ± 0.01 mm in VPA5 to 0.53 ± 0.08 mm in CONo1 (Fig. 1*A*). WM displayed lower average LT (0.44 ± 0.02 mm) than that of CON (0.38 ± 0.01 mm). There were no significant differences between CON and WM AGs (Table 1).

Leaf density (LD) values ranged between 105.1 ± 10.0 g dm^{-3} in CPA9 and 208.3 ± 17.4 g dm^{-3} in BRA1 (Fig. 1*B*). On average, the CON accessions displayed significantly lower LD (148.6 ± 5.5 g dm^{-3}) than that of the WM accessions (173.5 ± 4.0 g dm^{-3}). There were also significant differences between AGs averages, being CONo and CONt significantly lower than that of CONm and the WM AGs (Table 1).

Leaf mass area (LMA) ranged between 47.5 ± 3.6 g m^{-2} in BRA6 and 81.2 ± 3.3 g m^{-2} in BRA1 (Fig. 1*C*). No significant differences were found between CON and WM. CONm had higher LMA average (68.8 ± 2.4 g m^{-2}) than the remaining CON AGs, and also than CPA (59.9 ± 1.6 g m^{-2}). The latter AGs also had significantly lower average than the other two WM AGs (Table 1).

Net photosynthetic rate (P_N) ranged from 25.2 ± 0.6 µmol m^{-2} s^{-1} in CPA8 to 34.7 ± 1.9 µmol m^{-2} s^{-1} in VPA2 (Fig. 2*A*). On average, WM displayed significantly lower P_N than CON (Table 1). This was mostly due to the significantly lower average of CPA (27.6 ± 0.5 µmol m^{-2} s^{-1}) as compared to any other CON AGs and also to VPA (31.0 ± 0.9 µmol m^{-2} s^{-1}) (Table 1).

Stomatal conductance (g_s) ranged from 0.35 ± 0.01 mol m^{-2} s^{-1} in CPA8 to 0.60 ± 0.07 mol m^{-2} s^{-1} in CONo3 (Fig. 2*B*). WM had significantly lower g_s than CON.

Table 1. Leaf morphological and photosynthetic characterization of the different accession groups (AGs). CON − the control accessions group, including modern inbreeds (CONm), old varieties (CONo) and traditional varieties (CONt). WM − the Western Mediterranean accessions, including Balearic Islands Ramellet accessions (BRA) accessions, and Catalonian (CPA) and Valencian Country (VPA) Penjar accessions. Net photosynthetic rate (P_N), stomatal conductance (g_s), and mesophyll conductance (g_m) under ambient [CO_2], maximum velocity of Rubisco carboxylation (V_{cmax}), intrinsic water-use efficiency (WUE_i), leaf carbon isotopic composition (leaf $\delta^{13}C$), leaf mass per area (LMA), leaf thickness (LT) and leaf density (LD).Values are means (± SE) of 3–5 measurements per accession (n = 13 for CONm, 11 for CONo, 16 for CONt, 28 for BRA, 35 for CPA, and 21 for VPA). Two nested analyses of variance (nested *ANOVA*) were performed to test for differences among AGs and between CON and WM accessions, respectively. *Different letters* denote statistically significant differences among AGs, and asterisks between CON and WM. In both cases, significant differences between means were revealed by *Duncan*'s analysis (P<0.05).

AG	LT [mm]	LD [g dm^{-3}]	LMA [g m^{-2}]	P_N [µmol(CO_2) m^{-2} s^{-1}]	g_s [mol(H_2O) m^{-2} s^{-1}]	g_m [mol(CO_2) m^{-2} s^{-1}]	V_{cmax} [µmol(CO_2) m^{-2} s^{-1}]	WUE_i [µmol(CO_2) mol^{-1} (H_2O)]	Leaf $\delta^{13}C$ [‰]
CON	0.44 ± 0.02*	148.6 ± 5.5*	62.4 ± 1.4	31.0 ± 0.5*	0.49 ± 0.02*	0.33 ± 0.01	294.2 ± 5.8*	66.0 ± 1.9	−28.8 ± 0.1
WM	0.38 ± 0.01	173.5 ± 4.0	63.4 ± 1.3	29.1 ± 0.4	0.45 ± 0.01	0.33 ± 0.01	274.8 ± 3.8	66.0 ± 1.0	−28.8 ± 0.1
CONm	0.40 ± 0.02^{ab}	173.8 ± 9^{a}	68.8 ± 2.4^{a}	32.0 ± 1.0 ^{a}	0.50 ± 0.04^{a}	0.36 ± 0.03^{a}	300.4 ± 10.0^{a}	68.1 ± 4.1^{a}	−28.5 ± 0.1 ^{a}
CONo	0.46 ± 0.04^{a}	140.2 ± 8.8^{b}	59.6 ± 2.3^{b}	30.9 ± 1.0^{a}	0.48 ± 0.04^{a}	0.31 ± 0.01^{a}	288.8 ± 10.1^{a}	67.2 ± 3.7^{a}	−29.1 ± 0.2 ^{c}
CONt	0.45 ± 0.02^{a}	133.5 ± 6.9^{b}	58.7 ± 1.4^{b}	30.4 ± 0.8^{a}	0.49 ± 0.03^{a}	0.32 ± 0.02^{a}	293.0 ± 10.1^{a}	63.5 ± 2.5 ^{a}	−28.9 ± 0.1 ^{bc}
BRA	0.37 ± 0.02^{b}	181.8 ± 7.7^{a}	66.2 ± 2.5^{a}	29.7 ± 0.6^{ab}	0.44 ± 0.02^{a}	0.36 ± 0.02^{a}	281.0 ± 6.5^{ab}	68.1 ± 1.7^{a}	−28.8 ± 0.1 ^{bc}
CPA	0.38 ± 0.01^{b}	165.1 ± 5.7^{a}	59.9 ± 1.6^{b}	27.6 ± 0.5^{b}	0.43 ± 0.01^{a}	0.30 ± 0.02^{a}	263.2 ± 5.5^{b}	64.2 ± 1.5^{a}	−28.7 ± 0.1 ^{ab}
VPA	0.38 ± 0.02^{b}	176.0 ± 7.4^{a}	65.3 ± 2.4^{a}	31.0 ± 0.9^{a}	0.48 ± 0.03^{a}	0.35 ± 0.02^{a}	285.5 ± 8.0^{ab}	66.2 ± 2.3^{a}	−28.9 ± 0.1 ^{bc}

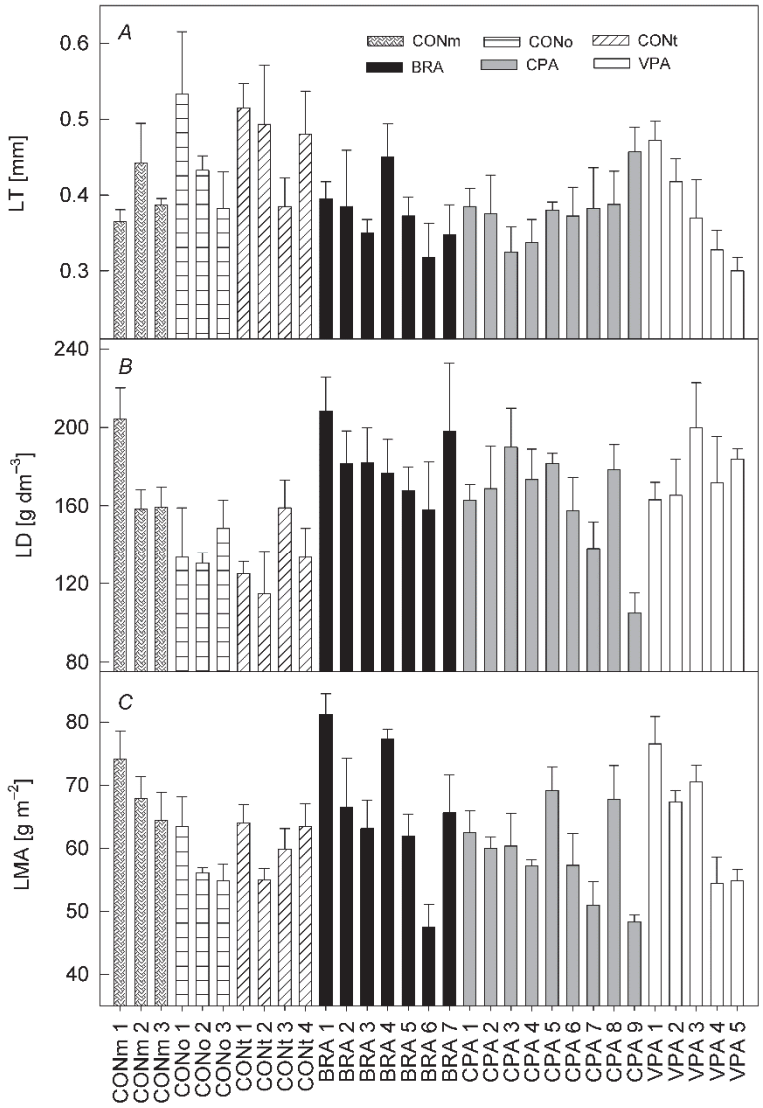

Fig. 1. Variability in (*A*) leaf thickness (LT), (*B*) leaf density (LD), and (*C*) leaf mass area (LMA) among the studied accessions. Data are means (n = 3–5) + SE. For labels *see* the text table in Materials and methods section.

No differences were found between CON and WM AGs (Table 1).

Mesophyll conductance (g_m) varied from 0.23 ± 0.02 mol m^{-2} s^{-1} in CPA9 to 0.46 ± 0.06 mol m^{-2} s^{-1} in CONm3 (Fig. 2*C*). There were no significant differences between CON and WM neither between AGs (Table 1).

Regarding to V_{cmax}, values ranged from 239.6 ± 13.9 µmol m^{-2} s^{-1} in CPA6 to 330.0 ± 3.8 µmol m^{-2} s^{-1} in CONt1 (Fig. 2*D*). WM presented significantly lower average values than CON. This was mostly the result of the significantly lower CPA average (263.2 ± 5.5 µmol m^{-2} s^{-1}) as compared to any CON AG (Table 1).

Intrinsic water-use efficiency (WUE_i) varied from 54.5 ± 2.1 µmol mol^{-1} in CPA5 to 76.9 ± 5.5 µmol mol^{-1} in BRA6 (Fig. 3*A*). There were no significant differences between CON and WM and neither between any AGs (Table 1).

For the leaf $\delta^{13}C$ composition, values ranged from –30.0 ± 0.2‰ in CONo1 to −28.0 ± 0.1‰ in CPA4 (Fig. 3*B*). No differences were found between CON and WM. The minimum and maximum AG average values were both found within CON (CONm, –28.5 ± 0.1 ‰ and CONo, –29.1 ± 0.2 ‰), with no differences for WM AGs (Table 1).

Determinants of the observed variability in the photosynthetic capacity and water-use efficiency among the tomato accessions: When considering all accessions together, positive relationships were found between P_N and g_s (R=0.71, P<0.001) and g_m (R=0.70, P<0.001) (Fig. 4*A,B*). Total conductance to CO_2 (g_{total}) was highly explicative of P_N (R=0.91, P<0.001, Fig. 4*C*).

As for the parameters related to biochemical traits, P_N correlated with ETR (R=0.69, P<0.001; Table 1S, *supplement available online*), but not with V_{cmax} (P<0.05, Fig. 4*D*), despite the relationship between ETR and V_{cmax} was significant (R=0.69, P<0.001; Table 1S).

No relationship was found between WUE_i and leaf $\delta^{13}C$ composition (P>0.05, Fig. 5*A*). Instead, WUE_i was positively related to g_s and g_m ratio (R=0.72, P<0.05, Fig. 5*B*).

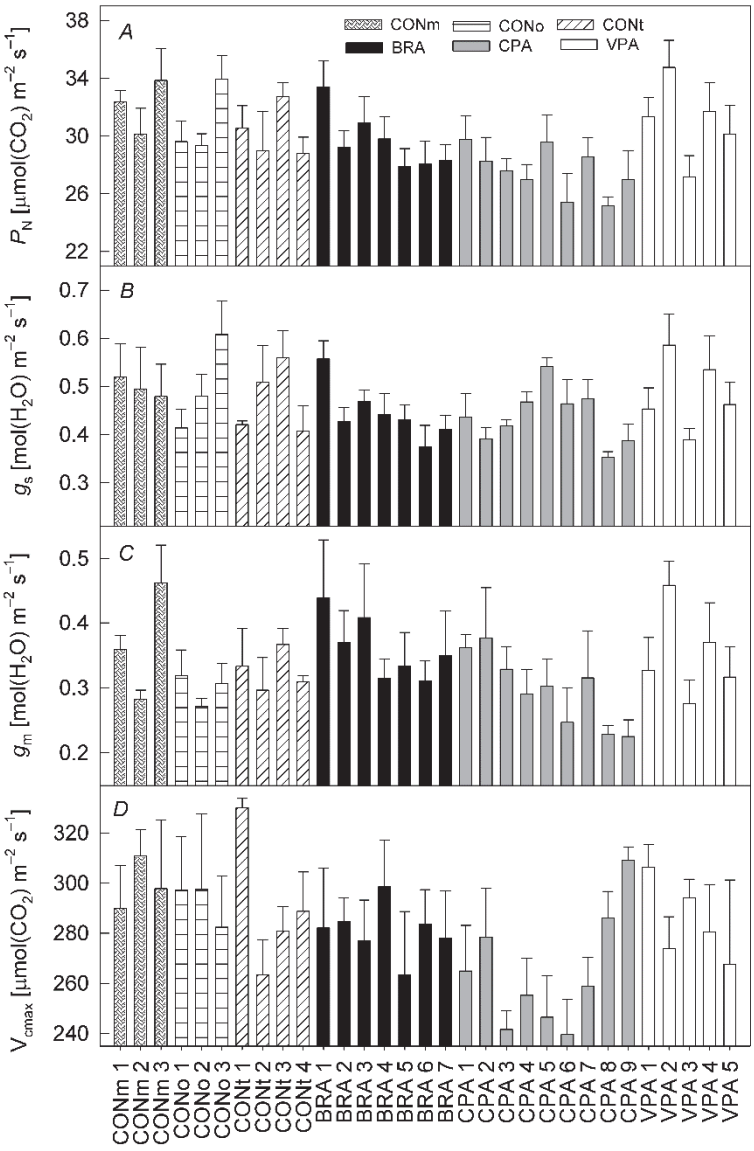

Fig. 2. Variability in (*A*) the photosynthetic rate (P_N), (*B*) the stomatal conductance (g_s), and (*C*) the leaf mesophyll conductance (g_m) under ambient [CO_2], and (*D*) the maximum velocity of Rubisco carboxylation (V_{cmax}) among the studied accessions. Data are means (n = 3–5) + SE. For labels *see* the text table in Materials and methods section.

Grouping of the tomato accessions based on the observed variability: To further differentiate the WM accessions, a cluster analysis was performed without considering the CON accessions. According to this cluster, the WM accessions were distributed in three major clades (Fig. 6). The first, most diverging clade included all CPA accessions but three, plus VPA5 and a BRA5. The second and third clades included BRA and VPA accessions and the three remaining CPA accessions (CPA2, CPA8, and CPA9).

A principal components analysis (PCA) was performed with including all accessions. The first two principal components (PCs) accounted for *ca*. 60% of the total variation. PC1 (33% of total variation) mostly represented the diffusive parameters (*i.e.* P_N, g_s, g_m, and C_c), while the biochemically related parameters (*i.e.* V_{cmax} and ETR) were mostly included in PC2 (29 % of total variation), which was mainly determined by WUE_i and LT. In the PC1 *vs*. PC2 plot (Fig. 1S, *supplement available online*), the ranges of BRA, VPA, and the CON accessions were almost completely overlapped, being VPA the most dispersed, whereas the CPA accessions were located in a narrower range concentrated in the positive axis of both PCs. Regarding to the remaining PCs and parameters, the major contribution of the leaf $\delta^{13}C$ composition, g_m, and LT was similarly distributed in PC3, PC4, and PC5, all together explaining for 30% of the variation.

Discussion

Leaf morphology and photosynthetic parameters discriminated among AGs: The spanning of key leaf morphological (LT, LD, and LMA) and photosynthetic traits (P_N, g_s, g_m, V_{cmax}, WUE_i, and leaf $\delta^{13}C$ composition)

indicated a large variability within each AG (Figs. 1, 2, 3). For most of these parameters, the intragroup variability was higher than that observed among the groups, either in the comparison between WM and CON, and among AGs within WM and within CON. This scenario could be expected based on the genetic similarity within cultivated tomatoes (*e.g.* Labate *et al.* 2009), the intrinsic plastic variation in most of the measured parameters (*e.g.* Hanba *et al.* 2002, Valladares *et al.* 2007, Flexas *et al.* 2013, Freschet *et al.* 2013), and the heterogeneity existing in the WM AGs (Penjar: Casals *et al.* 2012; Ramellet: Bota *et al.* 2014) and among the selected CON AGs.

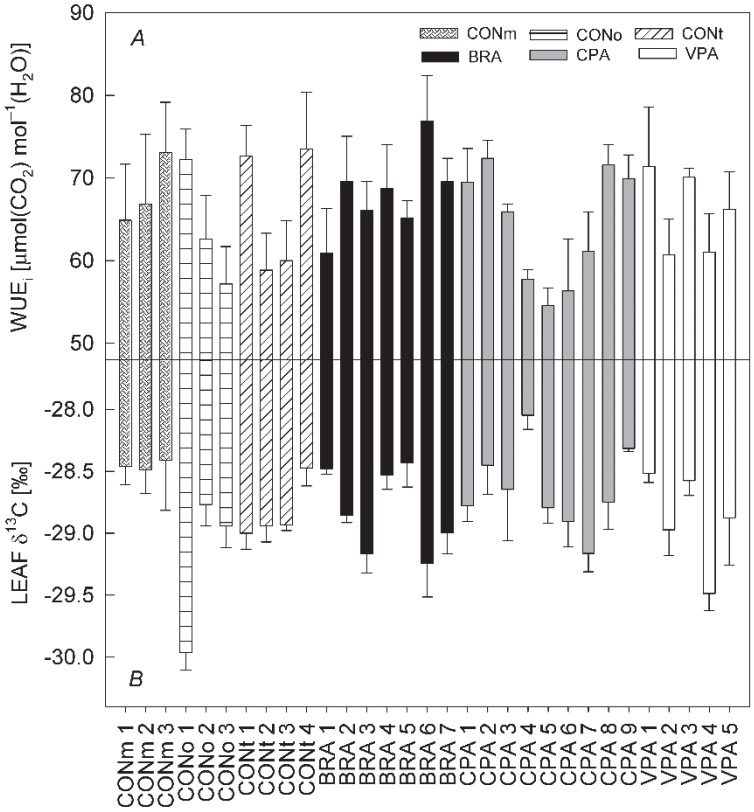

Fig. 3. Variability in (a) the intrinsic leaf water-use efficiency (WUE_i) and (b) the leaf carbon isotopic composition (Leaf $\delta^{13}C$) among the studied accessions. Data are means (n = 3–5) ± SE. For labels *see* the text table in Materials and methods section.

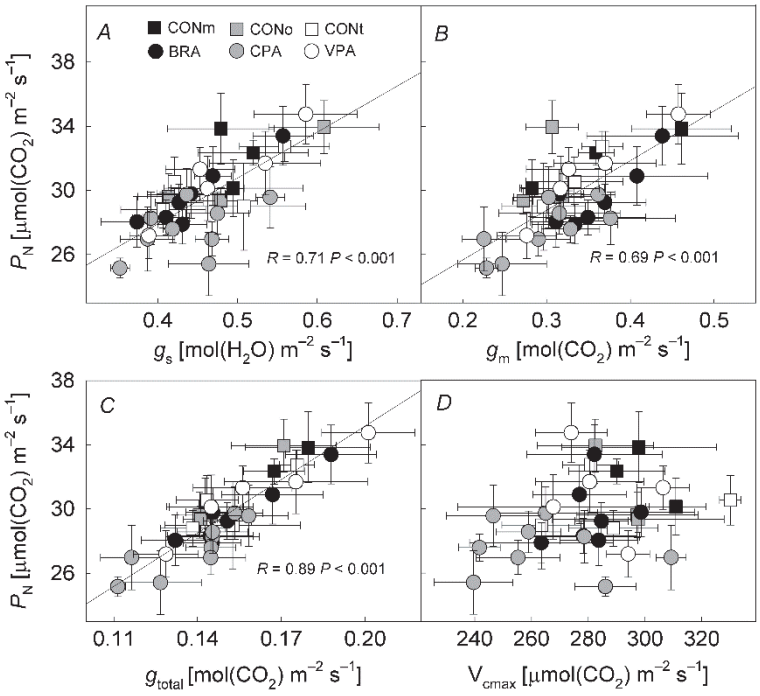

Fig. 4. The relationship between the net CO_2 assimilation (P_N) and (*A*) the stomatal conductance to H_2O (g_s), (*B*) the leaf mesophyll conductance to CO_2 (g_m), (*C*) the leaf total conductance to CO_2 (g_{total}) under ambient [CO_2], and (*D*) the maximum velocity of Rubisco carboxylation (V_{cmax}). Regression analyses considered all accessions together and are shown when significant. Data are means (n = 3–5) ± SE. For labels *see* the text table in Materials and methods section.

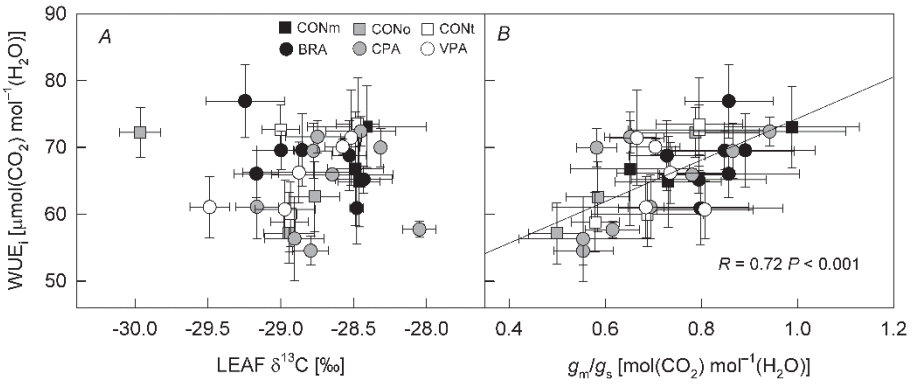

Fig. 5. The relationship between the intrinsic water-use efficiency (WUE_i) and (*A*) the leaf carbon isotope composition (leaf $\delta^{13}C$), and (*B*) the stomatal to leaf mesophyll conductance ratio (g_s/g_m). Regression analyses correspond to all accessions together. Data are means (n = 3–5) ± SE. For labels *see* the text table in Materials and methods section.

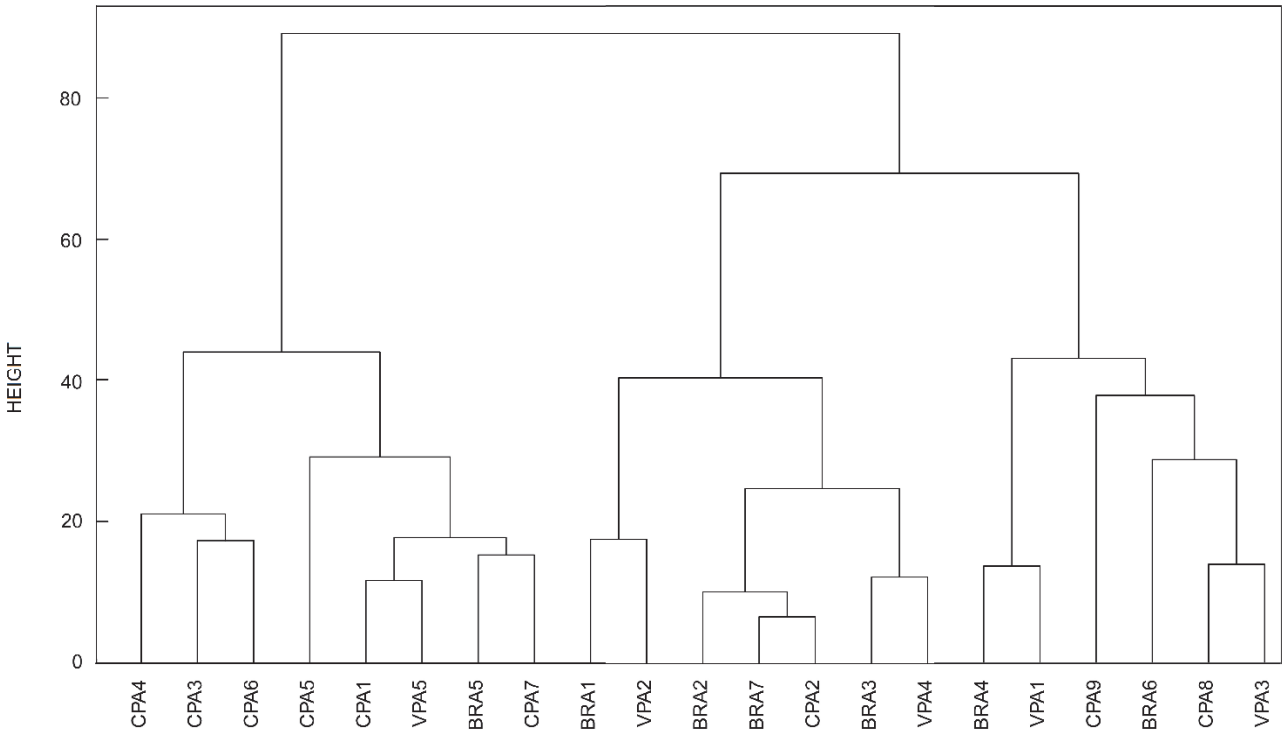

Fig. 6. Dendrogram resulting from the cluster analyses based on the following parameters: net photosynthetic rate (P_N), stomatal conductance (g_s), rate of mitochondrial respiration at darkness (R_D), mesophyll conductance (g_m), and chloroplastic CO_2 concentration (C_c) under ambient [CO_2], maximum velocity of Rubisco carboxylation (V_{cmax}), electron transport rate (ETR), intrinsic water-use efficiency (WUE_i), leaf carbon isotopic composition (leaf $\delta^{13}C$), leaf thickness (LT), and leaf mass per area (LMA) for the Western Mediterranean (WM) accessions. For labels *see* the text table in Materials and methods section.

Despite this, significant differences were found between CON and WM averages in two morphological traits (LT and LD) and three photosynthetic parameters (P_N, g_s, and V_{cmax}), (Table 1). Thus, CON had significantly thicker and less dense leaves than that of WM, as a result of the significantly higher LT and lower LD of CONo and CONt, as compared to any WM AG. These findings supports previous results of Galmés *et al.* (2013), reporting lower LT in Ramellet tomato accessions as compared to other tomatoes under well-watered conditions. Nevertheless, CONo and CONt were expected to be more similar to the WM AGs than to CONm due the different selection criteria. Contrarily, this AG had leaf morphology values closer to the WM AGs than to the remaining CON AGs (Table 1).

As reported for other species (Hanba *et al.* 1999, White *et al.* 2005, Marenco *et al.* 2009, Blonder *et al.* 2013), in our study, LT and LMA correlated positively (R=0.38, P<0.001; Table 1S), so that increments in LMA could be consequence of increased LT. Despite this, no differences were observed between CON and WM for LMA. This can be attributed to the large differences between CON AGs, since the average LMA of CONm was lower than those of CONo and CONt, while CPA had the lowest LMA

average, closer to CONo and CONt rather than to BRA and VPA (Table 1). Therefore, low LD of CONo and CONt compared to WM was the result of both low LMA and high LT, while a rather inverse trend arose in CONm, with higher LD resulting from higher LMA and lower LT. In turn, within WM, the significantly lower LMA of CPA was related to differences in LD rather than LT (Table 1).

Commercial cultivars have been selected for maximization of a plant yield through increased photosynthetic capacity and efficient allocation of carbon and energy to fruits (Bai and Lindhout 2007, Hajjar and Hodgkin 2007). Accordingly, CONm accessions (especially CONm3) were among those presenting the highest P_N. Although, despite CONt and WM accessions may have been selected for other traits rather than productivity, in both cases there were accessions with high P_N values (*e.g.* CONt3, and also BRA1, BRA3, and most of VPA accessions).

Regarding to photosynthetic traits, the higher V_{cmax} AGs averages corresponded to the higher P_N averages and *vice versa* for the lower AGs averages. The similarity between both parameters could indicate that P_N was limited by V_{cmax} (*i.e.* biochemical capacity). In other studies, higher biochemical capacity (or V_{cmax}) results from increased Rubisco concentration due to higher allocation of N in leaves (Centritto and Jarvis 1999). Nevertheless, the relationship of V_{cmax} with the Rubisco concentration has been proved to be the opposite and genotype-dependent in wheat (Driever *et al.* 2014).

Nevertheless, in addition to biochemical limitations, photosynthetic rates may also be limited by CO_2 diffusional limitations from the stomata to the carboxylation site (Sharkey 1985). Differences in g_s were also significant between CON and WM. Thus, as occurred for V_{cmax}, the higher AGs average values for g_s corresponded to the higher AGs averages values of P_N. Moreover, when comparing AGs, the higher g_m average also corresponded to the higher P_N average values, while the lower g_m average to the lower P_N average (Table 1). Thus, P_N limitations could be alternatively related to diffusive parameters.

Considering that CPA presented the lowest LMA within WM, the lowest P_N could be a consequence of this AG having less cell layers per unit leaf area (Flexas *et al.* 2008) but, however, this was not reflected in the LT. Thus, since LMA and g_m are in general negatively correlated (Flexas *et al.* 2008), the low P_N might be a consequence of further traits leading to lower g_m despite having lower LMA. Nevertheless, the correlation between LMA and g_m tended to be positive (Table 1S). In fact, a positive relationship between both traits was already observed in Ramellet in the acclimation to drought stress, where the LMA increase appeared to be consequence of the leaf thickness increase globally leading to a leaf density decrease (*i.e.* higher aerial space) which, in turn, led to a g_m improvement (Galmés *et al.* 2011; 2013). Similarly, the opposite to CPA can be stressed within CON for CONm, with the highest LMA and P_N and significantly the lowest LT. Therefore, it appears that the P_N improvement in the modern inbreeds might be also related to g_m improvements instead of LT.

Accordingly to the above, the cluster analysis (Fig. 6) tended to split CPA from the remaining WM AGs, which did not occur within CON (data not shown).

Thus, as previously observed in *Capsicum chilense* (Rosado-Souza *et al.* 2015), morphological and physiological differences can be at least partially related to the different geographic origins of the studied tomato landraces. Nevertheless, many physiological trait relationships did not follow the expected general trends because of the limited genetic variation and the large plasticity in most of the measured traits.

Which parameters determined the observed variability? According to the PCA analysis, P_N was among the most explicative parameter of the observed variation (Fig. 1S). Considering all accessions individually, a significant correlation of P_N was found with g_s and with g_m, and especially g_{total} (Fig. 4*A,B,C*). The latter was in agreement with previous reports in tomato (Galmés *et al.* 2011; 2013) and in other species (Evans 1999, Centritto *et al.* 2003, Soolanayakanahally *et al.* 2009, Flexas *et al.* 2014). Additionally, there was a lack of significant correlation between P_N and V_{cmax} (Fig. 4*D*). Contrary to the AGs averages, where the limitations of photosynthesis remained elusive, the correlations at the accession level indicate that photosynthetic capacity was limited by CO_2 diffusion rather than biochemical-related traits (Cornic 2000, Centritto *et al.* 2003, Galmés *et al.* 2013). This may be indicative that improvements in the photosynthetic capacity of tomato would come from increases in leaf CO_2 conductances, and that further increments in V_{cmax} (*e.g. via* enhanced N or Rubisco concentration in leaves) would not translate into higher P_N.

Moreover, when accounting for the diffusive limitations of P_N in well-watered crops, the importance of g_m and g_s has shown to be species-dependent. Thus, while both conductances are co-limiting in rice, and g_m appears as the major constraint in grapevine, the general trend across tomato cultivars worldwide appears to be limited by g_s (Flexas *et al.* 2013). Nevertheless, both g_m and g_s (and also P_N) values observed in any average AG are larger than the reported for tomato (*cf.* Flexas *et al.* 2013), and higher than the previously reported for Ramellet under well-watered conditions (Galmés *et al.* 2011). Therefore, the studied accessions showed a *ca.* 2–fold variation in both conductances to CO_2, even within each AG and thus, these accessions appear as valuable genetic resources to explore in the future in order to breed for diffusive limitation improvement.

The distinctive behavior of g_s and g_m in Ramellet grown under drought was related to anatomical traits of stomata and leaf mesophyll (Galmés *et al.* 2013). A future inspection of the anatomic determinants of g_s and g_m may provide some insights on the regulation of water and CO_2

fluxes in tomato leaves, and eventually provide some clues towards the enhancement of the CO_2 assimilation capacity of tomato plants.

The importance of leaf CO_2 conductance governing the photosynthetic performance of tomato leaves indicated above was further corroborated by the significant relationship between WUE_i and g_m/g_s (Fig. 5*B*). This relationship shows how those accessions with increased g_m to g_s ratio enhanced efficiently WUE_i, in agreement with past reports in a variety of species including tomato (reviewed in Flexas *et al.* 2013). Furthermore, and contrarily to several previous reports (*e.g.* Blum 2005), no correlation was found between WUE_i and P_N (Table 1S), suggesting that improving WUE_i may not necessarily result in decreased P_N and productivity, at least when this is achieved by improving g_m over g_s. In fact, the ordination of average values for WUE_i showed more similarity with that of g_m than with P_N or g_s (Table 1), which suggests that actually g_m is playing an important role in defining WUE_i and its lack of relationship with P_N in the WM accessions.

Contrary to WUE_i, significant differences were observed for the time-integrated leaf $\delta^{13}C$ measurements of water-use efficiency (Table 1), although only between CONo and CONm. Since $\delta^{13}C$ is generally conditioned by the environmental conditions (*i.e.* changes in C_i/C_a and C_c/C_a) (Seibt *et al.* 2008), this finding did not support the hypothesis that WM accessions, typically grown under non-irrigated conditions during the Mediterranean summer, presented an improved WUE compared to other nonWM or modern cultivars, at least when grown under unstressed conditions. On the other hand, our results (Fig. 5*A*) did not show the often observed tight and negative correlation between WUE_i and leaf $\delta^{13}C$ (Brugnoli and Farquhar 2000, Barbour *et al*. 2010 and references therein). This discrepancy has already been reported in other crops (*e.g.* Bota *et al*. 2016), and it could be due to modifications in the g_m (Parry *et al.* 2005, Warren and Adams 2006, Flexas *et al.* 2008, Seibt *et al.* 2008, Soolanayakanahally *et al*. 2009, Bickford *et al.* 2010). Indeed, this discrepancy has been related to differences in the main driver of WUE_i. Thus, when it is mainly determined by P_N the relationship is negative, whereas the lack of correlation has been related to cases when WUE_i differences are mainly driven by g_s (Farquhar *et al.* 1989). The lack of correspondence between both WUE measurements points to a major effect of g_s, g_m or both conductances to CO_2 in determining P_N in the WM tomato accessions.

Conclusion: High variation was found among the accessions in all the measured parameters of this study. Moreover, differences were found in leaf morphology and photosynthetic traits between WM and CON accessions and mostly AGs. According to gas-exchange results, P_N was limited by diffusive parameters (*i.e.* g_s, g_m, and combination of both – g_{total}) rather than by biochemical-related parameters (*i.e.* V_{cmax}). This must be considered in relation to future tomato improvement for cultivation outdoors under conditions similar to the Mediterranean summer. Nevertheless, despite the different selection criteria between WM and CON AGs, insignificant differences were found between the groups for WUE_i under the commercial growth conditions of this experiment.

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