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Additional Information

Exploring alternative germplasm for the development of stable high vitamin C content in tomato varieties.

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ABSTRACT

The genotypic potential of 15 accessions of *S. lycopersicon* and *S. pimpinellifolium* has been evaluated for high L-ascorbic acid content in three environments, including open-air and glasshouse cultivation and two localities. The environmental effect on L-ascorbic acid accumulation was highly genotype dependant and the variance ascribed to the GxE environment was similar in importance to that ascribed to genotype. The variation within accession might be ascribed to microenvironmental effects associated with temperature changes and oxidative stress or population variability. Several accessions with high genotypic values ($\mu+G$) have been identified. Among them, accession PI365959 of *S. pimpinellifolium*, showed a genotypic value of 293.8 mg kg⁻¹, statistically significant higher ($p<0.05$) than the genotypic potential of controls reported to have high L-ascorbic content (CDP4777, 115.0 mg kg⁻¹). It also showed positive GxE interactions, with a relatively high stability. Accession LA1423 of *S. lycopersicon* var. *cerasiforme*, despite being less stable, had also high genotypic values for L-ascorbic acid accumulation (197.4 mg kg⁻¹). This germplasm will be of great interest for the development of new tomato

cultivars targeted to added-value markets appreciating nutritional or functional quality. The close relationship of the selected material with the cultivated tomato will enable an efficient and rapid exploitation of their potential in breeding programmes.

Highlights:

>Germplasm from tomato and the closely related species *S. pimpinellifolium* has been evaluated for vitamin C content in three environments.>Environment effect on L-ascorbic acid accumulation was genotype dependent.> Accession PI365959 doubled genotypic potential of previously described high content controls, relatively high stability and positive environmental interactions.

KEYWORDS: FUNCTIONAL QUALITY, ASCORBIC ACID, GERMPLASM, TOMATO BREEDING

1. Introduction

In tomato (*Solanum lycopersicon* L.), as in other crops, the main breeding objective has been to develop cultivars with higher marketable productions, either increasing yield potential or reducing yield losses and extending shelf life. The results in yield improvement have been striking, but collateral effects have been detected, such as important negative consequences on tomato flavour (Goff and Klee, 2006). Though the introgression of pest and disease resistance genes still remains the main goal of numerous tomato breeding programmes, a new perspective is arising and improved organoleptic and nutritional or functional quality in the new cultivars is also sought (Dorais *et al.*, 2008). In this sense, consumers demand new cultivars with the traditional tomato flavour lost in modern varieties. Additionally, markets, especially in Western countries, are

demanding foods that improve health or help to prevent different diseases (Granato *et al.*, 2010).

In the case of tomato, its nutritional and functional quality is determined mainly by the accumulation of antioxidant compounds. Among these, the carotenoids lycopene and beta carotene, vitamin C (L-ascorbic acid) and flavonols are the most important. In the case of vitamin C, the contents registered in tomato are not especially high (Gould, 1992) and other fruits and vegetables such as orange or broccoli are renowned as more efficient sources of this vitamin (Davey, 2000). Nevertheless, despite not having high levels, tomato is still an important source of L-ascorbic acid, as its high volume of consumption all the year round (reaching 40-50 kg per capita and year in countries such as Spain, Italy or USA, *source: FAO databases*) makes this fruit one of the main sources for this vitamin. Wild relative species such as *Solanum peruvianum* L., *Solanum pennellii* Correll or *Solanum pimpinellifolium* L. have been found to be good sources of high ascorbic acid content (Stevens and Rick, 1986, Di Matteo *et al.*, 2010, Roselló *et al.*, 2011). In fact, cultivars such as Doublerich, derived from *S. peruvianum*, have been developed and commercialized as high vitamin C tomatoes. Nevertheless, their success has been limited. Among other factors, it has to be considered that vitamin C content is highly unstable, as it is very sensitive to environmental conditions (Hamner *et al.*, 1945; Hanson *et al.*, 2004) and as expected considering its antioxidant functions, the levels of ascorbic acid are responsive to stress factors (Davey *et al.*, 2000; Ioannidi *et al.*, 2009). Additionally, a deleterious effect of plant yield has been described for this type of high vitamin C cultivars due primarily to a reduction in fruit size (Stevens and Rick, 1986). Something similar applies to cultivars carrying the *high pigment* mutation, linked to high carotenoid and L-ascorbic acid contents that show important yield reductions (Stommel, 2006)

In this context, the purpose of this study was to find alternative germplasm sources of high and stable vitamin C content to be used in tomato breeding programmes. To achieve this objective, accessions from the cultivated species and its candidate ancestors have been tested in different environments to evaluate environmental effects and to detect the best genotypic potentials. These species were selected considering the easier introgression of traits from them into cultivated breeding lines and the lower interference of wild background in backcrossing programmes.

2. Materials and methods

2.1. Plant material

Two *Solanum lycopersicum* L. accessions (BGV5655 and BGV2091) with medium size and high number of locules, six *S. lycopersicum* L. with cherry type attributes (CDP4777, CATIE18628, BGV12627, LA3633, LA1423 and LA1289) and seven *Solanum pimpinellifolium* L. (BGV6195, PI365959, CATIE14812, PI503524, BGV6333, LA1781 and BGV4654) were studied. Accession LA3633 despite being classified as the former *cerasiforme* variety of *S. lycopersicum* in the TGRC database, showed intermediate traits between *S. lycopersicum* and *S. pimpinellifolium*. PI accessions were obtained from the Northeast Regional PI Station (U.S.), LA accessions were obtained from the Tomato Genetic Resource Center (TGRC, U.S.), CATIE accessions were obtained from Centro Agronómico Tropical de Investigación y Enseñanza (CATIE, Costa Rica), and CDP and BGV accessions were obtained from COMAV (Spain). Two breeding lines FORTUNA-C and NE-1 provided by COMAV (Spain) with standard to low vitamin C contents, previously used as controls in similar assays, were included as references. Accession LA-3538 from *S. lycopersicon* (provided by TGRC), which carries the *high pigment* gene (*hp¹*) was included as reference, as high ascorbic acid contents have been described in

this germplasm (Jarret *et al.* 1984). Accession CDP4777 was also included as it showed very high content in previous assays in the same conditions (Roselló *et al.*, 2011)

2.2. Experimental design and growing conditions

The trials were carried out in 3 growing environments representing common cycles and cultivation techniques. A randomized complete block design was used with 3 blocks per environment, 21 plots per block (one per accession) and 3 plants per plot.

Two sites of cultivation representing three environments were used during the spring-summer growing cycle. Cultivation at Castellón (39° 59' N, 0° 2' W 35m asl) was carried out in two different growing systems: in the open-air and in a glasshouse. Cultivation at Valencia (39° 28' N, 0° 22' W 15 m asl) was performed in a glasshouse. In protected cultivation heat dissipation systems (progressive shadowing and cooling) were used. Plants were staked and pruned properly and fertirrigation was applied following the usual regimes for the crop and area. Air temperature and photosynthetically active radiation (PAR) were recorded using WatchDog wheatear stations (Spectrum Technologies Inc., Illinois, USA) equipped with temperature, quantum light PAR sensors and data logger.

2.3. Sampling

Samples were obtained in a per plant basis from uniformly ripe, representative fruits, at the complete-ripe stage (when fruits reached maximum colour intensity). The fruits were harvested from the first 3 trusses to minimise intra-plant variability. In small fruited populations at least 10 fruits were sampled per plant, otherwise 4 fruits were sampled per plant. Samples were blended at low light intensity to minimise antioxidant loss and stored at -20°C until analysis.

2.4. Ascorbic acid determination

Ascorbic acid was quantified by Capillary Zone Electrophoresis using a P/ACE System MDQ (Beckman Instruments, Fullerton, USA), following the method described by

Galiana-Balaguer *et al.* (2001) with minor modifications. Two grams of sample were thawed in the dark at 4°C and centrifuged at 12500 rpm in a refrigerated centrifuge. The supernatant was diluted in 2% metaphosphoric acid to avoid ascorbic acid oxidation. Potassium hydrogen phthalate (100 mg l⁻¹) was used as an internal standard. Sample extracts were filtered through a 0.2 mm filter membrane (Millipore, Bedford, USA) prior to injection. Uncoated fused-silica capillaries (31.2 cm of total length, 21 cm of effective length, 50 µm i.d.) were used (Polymicro Technologies, Phoenix, USA). Hydrodynamic injection of samples was carried out at 0.5 psi during 5 s. Separation was performed at -15 kV and 25°C. Absorbance was measured at a detection wavelength of 254 nm. Each sample was analysed twice.

2.5. Statistical analysis

The mixed linear model used for the analysis of *i* genotype in *j* environment and *k* block inside environment *j* was:

$$Y_{ijk} = \mu + G_i + E_j + GE_{ij} + B_{k(j)} + e_{ijk}$$

Where *Y*=phenotypic value with population mean μ and variance V_P ; *G*=genotype effect with mean 0 and variance V_G ; *E*=environment effect with mean 0 and variance V_E ; *G_E*=Genotype x Environment interaction effect with mean 0 and variance $V_{G \times E}$; *B*=the block effect with mean 0 and variance V_B ; *e*=residual effect with mean 0 and variance V_e . All the factors were considered as random. The random effects were predicted using the adjusted unbiased prediction (AUP) method (Zhu and Weir 1996). Standard errors of the statistics were obtained by the jackknife procedures (Miller, 1974; Zhu and Weir 1996) and a two-tail t-test was performed for testing the significance of parameters obtained. The model was also recalculated considering genotype as fixed factor for mean performance accession comparison computing using LSD method at $\alpha = 0.05$.

All the data analyses were performed with QGASStation (v. 1) software (Bioinformatics Institute, Zhejiang University, China).

3. Results

Considerable climatic differences were detected in the three environments evaluated (Fig. 1) with higher temperatures obtained in Castellón glasshouse (CG), higher radiation in Castellon open-air (CO) and a reduction of approximately 45% of radiation by glasshouse covers. A lower temperature regime was found in Valencia glasshouse (VG) compared to CG due to the higher efficiency of heat dissipation systems.

A wide range of L-ascorbic acid accumulation was observed among the varieties and environments tested and even within variety (Table 1). Considering the different trends of variation across the environments for phenotypic values it resulted obvious the existence of considerably high genotype x environment interactions.

A decomposition of the phenotypic variance showed that the environment and block effects were not significant ($p=0.1$ and $p=0.5$ respectively). The rest of the effects were significant ($p<2.5 \cdot 10^{-11}$). The estimate of genotypic variance (\pm standard error) represented a $28.7\pm 1.2\%$ of the global phenotypic variance, the genotype x environment interaction a $24.9\pm 1.3\%$ and the residual variance a $46.4\pm 1.2\%$. It should be noted that despite the lack of significance of the environmental effect, the environment showed an important effect through the genotype x environment interaction.

The estimates of the overlaying genetic model enabled the decomposition of the phenotypic effect in the relative significant contributions of total genotype ($\mu+G$), and each environmental interaction: CO, CG and VG. Fortuna C and NE-1, control lines with usually standard to low L-ascorbic acid values showed as expected the lower genotypic contributions and low environmental interactions (Fig. 2). Positive control LA-3538 showed a similarly low genotypic contribution and interactions. Finally positive control

CDP4777 showed the highest control genotypic contributions (almost exceeding in a 50% the genotypic potential of the rest of the controls), with higher values obtained at the environment VG.

Among the accessions tested only accession BGV5655 showed genotypic values as low as the lower controls. Accessions CATIE18628, CATIE14817, LA1289, ECU567, USAL31 and CL68 showed similar genotypic contributions to that of the positive control accession CDP4777 (Fig. 2).

Accessions BGV6195, BGV12627, LA3633, CATIE14812, PI503524 and LA1781 showed genotypic contributions moderately higher than the positive control (Fig. 2). Among them PI503524 showed a considerably high and positive interaction in the CO environment that, added to the mean and genotypic values, clearly exceeded the potential of the positive control CDP4777, though a considerably high and negative interaction in the VG environment was also recorded.

Finally the best genotypic contributions were recorded in accessions PI365959 and LA1423 (Fig. 2). In fact, the genotypic contribution of PI365959 (293.8 mg kg^{-1}) doubled the genotypic potential of the positive control CDP4777 115.0 mg kg^{-1} . LA1423 also had a high genotypic value (197.4 mg kg^{-1}), but also a considerably high and positive interaction in the VG environment but a relatively high and negative interaction in the CG environment. Conversely the accession PI365959 had positive interactions in both CO and CG environments even increasing the phenotypic potential and almost null but not negative extra contribution in the VG environment.

4. Discussion

Considering that a mean of 200 mg kg^{-1} has been described as the usual L-ascorbic acid content in fresh tomato varieties (Gould, 1992), the values obtained in this work may

seem not especially outstanding. Nevertheless, the environmental conditions of the growing cycle might have been limiting. Here relies the importance of the use of low and high content controls in this type of screening works. In our case, the low content controls have previously shown much higher levels close to those obtained in standard commercial tomato varieties, with NE-1 showing up to 162.4 mg kg⁻¹ in other campaigns (Galiana-Balaguer *et al.*, 2006). Additionally, the positive control LA3538, reached up to 189 mg kg⁻¹ in different years (Adalid *et al.*, 2008) and the remaining control CDP4777 showed previously mean phenotypic values between 250 mg kg⁻¹ and 346 mg kg⁻¹ depending on the environment considered (Roselló *et al.*, 2011).

In the present study the values obtained for these controls were considerably lower. These results confirmed that the environmental conditions in the three environments were limiting for L-ascorbic acid accumulation even in different locations, probably due to high temperatures related to its oxidation (Murneek *et al.*, 1954) and high radiation levels (Adegoroye and Jolliffe, 1987), though other unfavourable year effects should not be discarded. Nevertheless, this fluctuation is usual in compounds such as L-ascorbic acid, so prone to environmental effects. As an example, the cultivar DoubleRich developed from crosses with *S. peruvianum* has been reported to have contents from 318 mg kg⁻¹ (Watada *et al.*, 1976) to 500 mg kg⁻¹ (Stevens and Rick, 1986). Several aspects explain these large variation levels, with temperature and mainly sun radiation playing a predominant role in ascorbic acid accumulation (reviewed in Dumas *et al.*, 2003). Additionally, oxidative stresses may involve a consumption of L-ascorbic resources to scavenge ROS in post-hypoxic and post-anoxic stresses or temperature stresses (Ioannidi *et al.*, 2009). These effects joined to the genotypic variation typical of wild or traditional populations might also explained in a microenvironmental level the variation found within accession.

The lack of significance of the environmental effect obtained in the present study reinforces the idea that the most important effect of the environment on phenotypic values depends greatly on the genetic material being considered and therefore it is expressed through the genotype x environment interaction. In fact, the variance ascribed to the genotype x environment interactions has a similar level than the variance ascribed to the genotype, even using a wide diversity of germplasm.

Considering the limiting environmental conditions for the accumulation of L-ascorbic acid, and the values obtained for the controls, the phenotypic values obtained in the accessions evaluated are really interesting. As previously stated, the frequent oscillation of L-ascorbic contents makes of limited use the analysis of germplasm potential considering only phenotypic values. The decomposition of the phenotypic value in its significant components: the genotypic potential ($\mu+G$) and the genotype x environment interaction values, allowed the selection of potential sources of variation by their genuine value discarding spurious variation.

An especially outstanding value has been identified in the accession PI365959 of *S. pimpinelifolium*, not only by its significantly higher genotypic potential, but also for its proved stability in different environments, even with adverse environmental conditions including high radiation levels (even with cover reduction) and higher stressing temperatures. The high genotypic value of other accessions such as LA1423 of *S. lycopersicon* var. *cerasiforme* should not be neglected. Despite its lower stability, it is of great interest for specific environments, especially considering its closer relation to cultivated tomato. Both accessions had a genotypic value much higher than the positive controls, LA3538 that carries the *high pigment* mutation (*hp¹*) related to high contents of L-ascorbic acid (Jarret *et al.*, 1984) and CDP4777, previously studied in detail (Roselló *et al.*, 2011), showing a genotypic value 150% higher than the standard controls.

It should be noted that especially high values of L-ascorbic acid have been also detected in other related wild species such as *S. pennelli* (Di Matteo *et al.* 2010) or *S. peruvianum* (Stevens and Rick, 1986), but considering their genetic distance with cultivated tomato, and especially the great differences in fruit organoleptic quality, it is much more difficult to exploit these sources of variation. In this sense, in crosses of cultivated tomato with *S. pennellii*, the hybrids only increased a 20% of the wild parent potential (Di Matteo *et al.*, 2010). For that reason in this work only the variety cerasiforme of the cultivated species and *S. pimpinellifolium* were used, as these resources are the closest related to cultivated tomato and fruits present similar characteristics and thus, its potential its more easily capitalized.

5. Conclusion

Vitamin C content in tomato related germplasm was extremely variable, though the effect of environment depended greatly on the genotype and therefore G x E interaction played a relevant role in the configuration of the phenotypic value. A microenvironmental effect due to temperature differences or oxidative stress joined to genetic variation between individuals in the wild and traditional populations used in the study might be the cause of the observed within accession variation.

Two accessions PI365959 of *S. pimpinellifolium* and LA1423 of *S. lycopersicon* var. *cerasiforme* have shown a high genotypic potential for the improvement of vitamin C content in new varieties of tomato targeted to added value markets appreciating nutritional and functional quality. An extra value relies in accession PI365959, as it showed relatively high stability with positive interactions contributing to higher phenotypic values. The close relationship of both materials with cultivated tomato will enable a more efficient and rapid exploitation of their potential in breeding programmes.

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Table 1. Phenotypic mean content of L-ascorbic acid (mg kg^{-1} of fresh weight) in the accessions and environments evaluated (mean \pm standard error). S.L.: *Solanum lycopersicon*; S.L.c.: *Solanum lycopersicon* var. *cerasiforme*; S.P.: *Solanum pimpinellifolium*.

| Accession | Species | Environment | | |
|-------------|--------------|-----------------------|-------------------------|------------------------|
| | | Castellón Open-air | Castellón Glasshouse | Valencia Glasshouse |
| FORTUNA-C | S.L. | 61.2 \pm 2.0 | 65.9 \pm 5.2 | 91.7 \pm 14.1 |
| NE-1 | S.L. | 54.5 \pm 3.1 | 61.4 \pm 4.0 | 42.5 \pm 8.0 |
| LA3538 | S.L. | 63.1 \pm 8.1 | 49.3 \pm 1.8 | 54.8 \pm 14.0 |
| CDP4777 | S.L.c. | 94.1 \pm 10.1 | 73.5 \pm 8.2 | 174.8 \pm 21.1 |
| BGV6195 | S.P. | 87.7 \pm 2.0 | 212.5 \pm 6.9 | 144.2 \pm 23.3 |
| BGV5655 | S.L. | 66.2 \pm 2.1 | 95.9 \pm 3.4 | 65.8 \pm 16.7 |
| CATIE18628 | S.L.c. | 108.3 \pm 6.3 | 135.5 \pm 6.5 | 116.8 \pm 17.6 |
| BGV12627 | S.L.c. | 132.5 \pm 8.1 | 144.0 \pm 5.2 | 144.2 \pm 23.3 |
| LA3633 | S.L.c x S.P. | 138.3 \pm 5.0 | 149.9 \pm 10.5 | 221.9 \pm 53.7 |
| PI365959 | S.P. | 350.3 \pm 67.2 | 348.3 \pm 13.1 | 278.2 \pm 47.3 |
| LA1423 | S.L.c. | 182.2 \pm 16.5 | 103.7 \pm 4.3 | 335.7 \pm 32.6 |
| CATIE-14817 | S.L.c. | 106.5 \pm 4.5 | 121.0 \pm 6.8 | 114.7 \pm 30.0 |
| CATIE-14812 | S.P. | 120.8 \pm 8.7 | 126.5 \pm 6.2 | 213.9 \pm 41.4 |
| LA-1289 | S.L.c. | 112.1 \pm 5.3 | 144.0 \pm 6.6 | 136.5 \pm 27.4 |
| PI 503524 | S.P. | 273.3 \pm 40.1 | 214.4 \pm 1.2 | 37.9 \pm 9.8 |
| BGV6333 | S.P. | 194.6 \pm 7.2 | 154.4 \pm 3.7 | 50.4 \pm 10.3 |
| LA1781 | S.P. | 123.3 \pm 7.6 | 164.6 \pm 0.2 | 221.4 \pm 23.9 |
| BGV4654 | S.P. | 154.6 \pm 24.4 | 150.4 \pm 26.3 | 96.5 \pm 9.7 |
| BGV2091 | S.L. | Not available | 106.3 \pm 9.2 | 129.9 \pm 13.7 |

Fig. 1. Climatic conditions of the environments used for cultivation. CO: Castellón open-air, CG: Castellón glasshouse, VG: Valencia glasshouse, Tmax: daily maximum temperature, PARmax: daily maximum photosynthetically active radiation.

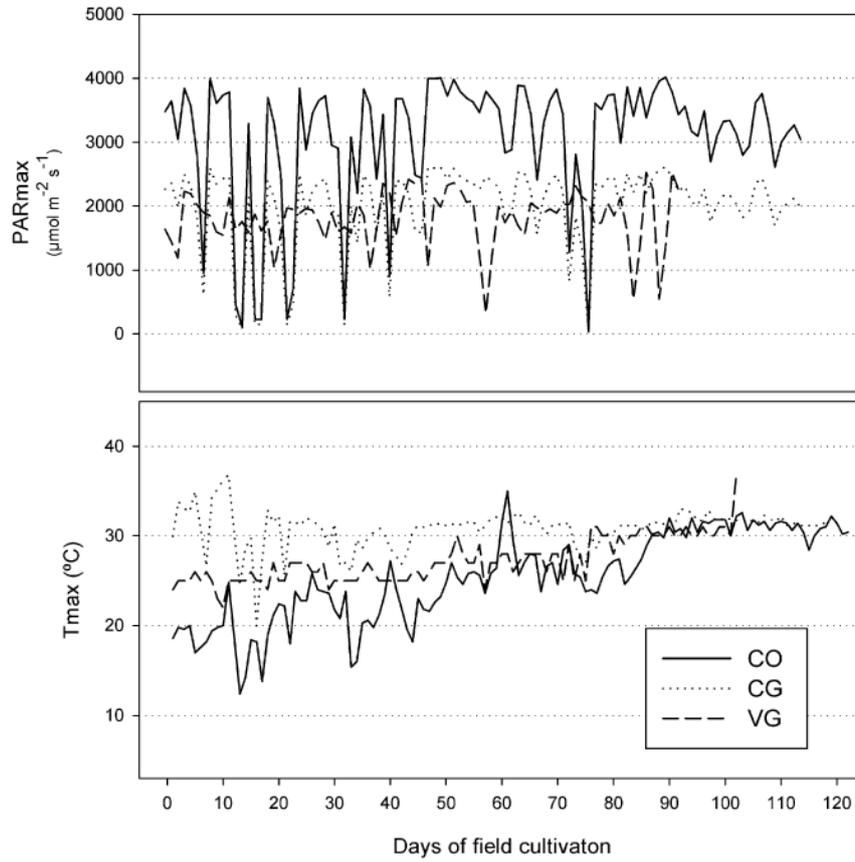


Fig. 2. Predicted total genotypic ($\mu+G$) and interaction effects ($G \times E$) for ascorbic acid content of the accessions studied. Controls in x-axis are inside a box. Reference lines: higher genotypic value of controls (upper dashed line), lower genotypic value of controls (lower dashed line). Environments E_1 : Castellón openfield; E_2 : Castellón glasshouse, E_3 : Valencia glasshouse. Different letters indicate significant differences in genotypic effect between accessions (LSD test, $p < 0.05$). For each accession estimated value significantly different from zero (t-test) indicated for different probabilities: +: $P = 0.1$, *: $P = 0.05$ and **: $P = 0.01$.

