Ascorbic Acid Distribution in Three Introgression Lines of Tomato

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Abstract

Total ascorbic acid (total-AsA) content and percentage of reduced ascorbic acid (AsA) on total-AsA were investigated and discussed in fruits, leaves, petiole, stem and roots of the *Solanum lycopersicum* cv M82 and *S. pennellii* introgression lines IL7-3, IL10-1 and IL12-4. In fruits, total-AsA content showed to be different according to genotype analysed. Higher total-AsA accumulations were observed for IL7-3 and IL12-4 followed by M82 and IL10-1. Total-AsA was generally higher in leaves than in petioles, stems and roots of all genotypes. In roots higher total-AsA concentration and lower AsA percentage was generally observed in introgression lines than M82, in particular for IL7-3.

Keywords: Antioxidant, Vitamin C, ILs, *Solanum lycopersicum*, *Solanum pennellii*

1. Introduction

In plant, ascorbic acid is a co-factor for many enzymes, contributes to detoxify reactive oxygen species and is important for resistance against biotic and abiotic stress, senescence regulation and floral induction (Arrigoni and De Tullio, 2002, Conklin and Barth, 2004, Barth *et al*. 2006, El-Gamal *et al*. 2007, Athar *et al*. 2008). Ascorbic acid is also implicated in biosynthesis and signalling of many plant hormones, controls stomata function and it is involved in photosynthesis, root development and nutrient uptake (Davey *et al*. 2000, Bloom *et al*. 2003, Chen and Gallie 2004, Athar *et al*. 2008). Ascorbic acid is synthesised through different biosynthetic pathways, mainly Wheeler-Smirnoff pathway that use L-Galactose as key intermediate, and L-Gulose pathway (Wheeler *et al*. 1998, Wolucka and Van Montagu, 2003). Many genes involved in these pathways have been identified (Conklin *et al*. 1999, Wolucka *et al*. 2001, Gatzek *et al*. 2002, Valpuesta and Botella 2004). In many plant tissues, the concentration of ascorbic acid and its oxidized forms (monohydroascorbate and dehydroascorbate) are tightly controlled and they depend on biosynthesis of ascorbic acid and its recycling by specific reductases.

In human diet the intake of antioxidants, such as ascorbic acid, by the consumption of fruits and vegetables is associated with a reduction of cardiovascular and cancer diseases, as well as other chronic disease (Hung et a1. 2004). The wide complex of antioxidants of such foods exhibits synergistic behaviour for a better therapeutic effect against pro-oxidant and anti-oxidant imbalances (Tiwari 2001). Thus, the selection of crops with high antioxidant concentration is an important goal. Further, the antioxidant recovery from by-products of a crop and processing industry could be of economical benefit for the food and pharmaceutical industries (Knoblich *et al*. 2005, Pacco *et al*. 2007).

Among vegetables, tomato (*Solanum lycopersicum*) is an important source of antioxidants. It is the main vegetable crop in the world (about 126 million tons fresh fruit) next to potato, and in some countries such as USA, fresh and processed tomato consumption shows an increasing trend (FAO Statistical Database, 2008). Tomato is the second source of vitamin C in human diet after orange and it contains remarkable concentration of other vitamins (folate and E), carotenoids (lycopene and β-carotene) and polyphenols (Beecher 1998).

Many researches have been conducted to improve antioxidant content in tomato fruit through genetic engineering and breeding schemes (Willits *et al*. 2005, Butelli *et al*. 2008, Sapir *et al*. 2008). Because the breeding schemes often require considerable investments to select and characterize genetic resources, the use of available genetic stocks, such as introgression line populations, is advantageous. Introgression lines (ILs) consist of plants harbouring single homozygous chromosomal segments that are introgressed from a wild parent in the genomic background of the cropped tomato (Eshed and Zamir 1995). ILs have been used for the identification and mapping of QTLs controlling traits of interest (Rousseaux *et al*. 2005, Schauer *et al*. 2006) and they represent a valuable tool for understanding the regulation of ascorbic acid (Barone *et al*. 2009). Tomato libraries have been developed for *S. pennellii* and studies on the primary metabolic traits in populations from interspecific cross with *S. lycopersicum* are available (Eshed and Zamir 1995, Schauer *et al*. 2006).

Although many ascorbic acid QTLs have been discovered in IL fruits, very little is known regarding ascorbic acid distribution in the whole plant in relation to fruit QTLs observed. Consequently, in this work the ascorbic acid profile was investigated in several organs collected from selected ILs of *S. pennellii* in *S. lycopersicum* cv M82 that, in our experimental condition, differed consistently in fruit ascorbic acid accumulation compared to M82 parental line. Such differences could be useful to evidence factors associated to the introgressions that can affect ascorbic acid distribution.

2. Materials and Methods

2.1 Plant material and morphological analysis

Three sets of three plants per genotype were grown in greenhouse (24/18°C day/night), in 5L pots filled with a mixture (1:1 v/v) of Vesuvian sandy soil and commercial substrate (professional substrate Type-S, FloraGard, Oldenburg, Germany), from November 2007 to flowering or red mature stages. At flowering stage, on one set, plant height, number of main stem nodes, number of the lateral shoots, leaf and leaflet areas, number of roots and their length were evaluated. Also dry weight (DW) was measured after dehydration at 70°C for 24 hours. Leaf morphological analysis was performed on two fully expanded leaves collected from node number 5 and 6. Leaf area was measured by image analyser software ImageJ 1.4 (Rasband 2005). At flowering stage, on a second plant set, ascorbic acid analysis was carried out on stem, petiole, distal and proximal leaflets sampled from two different sections: S1, from node 1 to node 4, and S2, from node 5 to node 8 (Figure 1). Ascorbic acid was determined also in roots collected and washed by running tap water to remove the soil. Fruits were harvested from the third plant set, grown from November 2007 to red mature stage, and their pericarp was separated. All plant samples were frozen immediately in liquid nitrogen and stored at -80°C.

2.2 Ascorbic acid extraction and quantification

Frozen tissue (250 mg) was homogenized in a 2 ml plastic tube using a Tissue Liser (Quiagen S.p.A., Milan, Italy) at 4°C, and ascorbic acid was extracted adding 0,2 ml of 6% (v/v) trichloroacetic acid (TCA) (Sigma-Aldrich S.r.l., Milan, Italy) for 10 minutes. After centrifugation (14,000 rpm, 20 min), supernatant was collected and diluted to 0.5 ml with TCA. Total ascorbic acid, reduced ascorbic acid plus monodehydroascorbate and dehydroascorbate (total-AsA), and reduced ascorbic acid (AsA) were determined according to Kampfelgen *et al*. (1995). The analysis were carried out three times

2.3 Statistical analysis

Statistical analysis was performed by using the Statistical Package for Social Sciences, version 14 (SPSS Inc Chicago, Illinois). Analysis of variance (ANOVA) was performed on ascorbic acid determination among vegetative tissues and among genotypes for each vegetative tissue and for fruits to evidence differences at 5% probability level. Student's t-test was applied to determine the differences ($p \leq 0.05$) of each morphological measurement between IL7-3 and M82 genotypes. The mean value for each determination and standard error were reported.

3. Results and Discussion

A preliminary study was performed on ascorbic acid accumulation in mature fruits of fifty *S. pennellii* introgression lines (ILs). By such investigation, we chose ILs that showed positive and negative differences in comparison to M82 parental line for three years (Di Matteo *et al*. 2009). The last year (2008) tissues from the whole plant were examined.

The total ascorbic acid (total-AsA), as sum of reduced (AsA) and oxidized forms, in the fruits of IL7-3 and IL12-4 showed to be higher than M82 parental line, while for IL10-1 it was lower than M82 although not statistically significant (Table 1). The increase of total-AsA for IL12-4 and the decrease for IL10-1 were in agreement with other authors (Rousseaux *et al*. 2005, Schauer *et al*. 2006); whilst the identification of IL7-3 as the highest total-AsA producer was in contrast to literature where no differences was found in this genotype as compared to M82 (Rousseaux *et al*. 2005, Schauer *et al*. 2006, Stevens *et al*. 2007). However, such result for IL7-3 was confirmed in our laboratory also in previous two years studies (Di Matteo *et al*. 2009). The discrepancy of ascorbic acid in IL7-3 fruit recorded in our study may arise by the choice of tissue used, which was unpeeled pericarp wall. Rousseaux *et al*. (2005) and Stevens *et al*. (2007) analysed entire fresh fruit whereas Schauer *et al*. (2006) peeled pericarp. However, also the environment highly influences ascorbic acid in fruits so that the genetic effects of single introgression may not be as strong as environmental ones (Toor *et al*. 2006). Moreover, many observed QTL in field were not always significant in different seasons and they were sometimes not confirmed in greenhouse (Rousseaux *et al*. 2005, Schauer *et al*. 2006, Stevens *et al*. 2007). It has been shown that IL7-3 produces fruits having higher sugar content on wet weight basis than M82 and this was attributed to the overexpression of genes involved in sucrose mobilization (Overy *et al*. 2005, Baxter *et al*. 2005). However, for *S. pennellii* ILs a significant year x genotype interaction has been ascertained for fruit weight and for sugar content on dry weight basis, but not for ascorbic acid (Stevens *et al*. 2007). Thus, another suggestion for the recorded increased of total-AsA in IL7-3 fruits on fresh weight basis may be a lower water content that unfortunately was not ascertained. Analogously, also the total-AsA increase observed in IL12-4 fruits may arise from lower water content.

The percentages of AsA on total-AsA detected in the fruits was similar among screened genotypes (Table 1) suggesting homogenous ripening level at harvest (Yahia Elhadi *et al*. 2001, Mondal *et al*. 2004).

The content of total-AsA was detected through the whole tomato plant and some differences in its distribution in vegetative tissues were found (Figure 2). Among tissues, total-AsA was generally higher in leaves than in petioles, stems and roots for all genotypes. This is because ascorbic acid is mainly produced in mature leaf to be translocated by phloem to non-photosynthetic tissues for its primary role in cell division/growth (Tedone *et al*. 2004). Such distribution has been shown also in rice and apple leaves compared to stems and roots (Baba and Inada 1956, Zadeh *et al*. 2007). In general, total-AsA did not change significantly between proximal and distal leaflets of the same leaf except for some differences for M82 and IL12-4. Total-AsA frequently showed lower values in top leaflets as compared with the bottom leaflets, also with some significant differences for IL7-3. This could be related to the full photosynthetic maturity of bottom leaves and/or to a higher transport of ascorbic acid from the top leaves that are closest to the floral sink (Franceschi and Tarlyn 2001). Generally, stem and petiole of all genotypes showed a similar total-AsA content. Furthermore, roots had total-AsA content similar to that of stem except for IL7-3 where it was higher.

Among genotypes, leaflets of each section did not show note worthy differences and petioles and stems evidenced some differences only in section one. IL roots, particularly for IL7-3, showed a higher total-AsA than

M82. It could be due to a probable higher number of apexes, generally richer in ascorbic acid (Cordoba-Pedregosa *et al*. 2003), in sampled root of ILs. In fact, IL7-3 roots (Table 2, Figure 3), and less markedly those of IL10-1 and IL12-4 (data not shown), were shorter and more ramified than those of M82. Furthermore, despite the altered root morphology of IL7-3 its root dry weight was similar to that of M82 (Table 2). Such different morphology could be attributed to the reduced vacuolar invertase activity (Sturm 1999, Sergeeva *et al*. 2006) reported for IL7-3 and IL12-4 (for IL10-1 invertase activity was not ascertained) (Baxter *et al*. 2005). In fact, in transgenic carrot plants with repressed invertase activity a decrease of plant taproot development has been observed (Tang *et al*. 1999). A support to this hypothesis was the significant increase of the number of leaves, due to higher lateral shoot development, with lower leaflet area that was recorded in IL7-3 (Table 2, Figure 3). This is in agreement with the higher number of narrower leaves observed in above-mentioned transgenic carrot (Tang *et al*. 1999). However, IL7-3 leaves showed a total leaf area similar to that of M82 because the presence of more developed intercilary leaflets. In any case, the lower dry weight of IL7-3 leaves (Table 2) was due to their lower thickness by touch. Although IL7-3 showed no increase of node number and lower plant height as compared to M82, both plants showed a similar total dry weight suggesting a similar ability of IL7-3 to fix carbon, which was distributed in higher leaf number due to ramification (Table 2). However, other authors have been observed an increase of weight of IL7-3 vegetative part of plants, on both dry and fresh weight (Eshed and Zamir 1995).

The percentage of AsA on total-AsA was highly variable among tissues and genotypes (Figure 4). About tissues of all genotypes, higher percentage of AsA was frequently observed in stems, petioles and roots than leaves. In stem this was probably because a reducing environment in phloem (Hayashi *et al*. 2000). The roots of IL genotypes showed lower percentage of AsA than M82. It has been demonstrated that lower percentages of reduced ascorbic acid stimulate root growth and nutrient assimilation (Bloom *et al*. 2003), further ascorbic acid supplied to roots enhances plant growth under water stress conditions (Athar *et al*. 2008). Thus the lower percentage of AsA in ILs (Figure 4), and the above-mentioned higher total-AsA (Figure 2), particularly for IL7-3, could be due to plant response to contrast the limiting effect of short root on soil exploration for nutrient and water uptake. Such limiting effect could be less important for IL12-4 and IL10-1 because less evident morphological difference (data not shown) than IL7-3.

A negative effect on plant growth of low invertase expression could be limited by the stimulation of invertase activity. It is known that such stimulation could be related to auxins increase (Mohsin and Naqvi 2007). Hence, the morphological differences observed, as well as ascorbic acid distribution, in the ILs could depend on hormone behaviour such as auxin synthesis/distribution. It has been shown that auxin influences both root morphology and relative content of ascorbic acid in tomato roots (Tyburski *et al*. 2008). Auxin increasing concentration decreases the percentage of reduced ascorbic acid because it stimulates ascorbate oxidase and ascorbate peroxidase activities and inhibits dehydroascorbate reducing enzymes; concomitantly, auxin reduces root elongation but increases lateral root formation (Tyburski *et al*. 2008). In fact, an increase in transport of indole acetic acid (IAA) and its accumulation in maize root tips caused an increase of ascorbate oxidase expression and activity, as well as a depletion of the reduced ascorbic acid in quiescent center (mitotically inactive cells within the root meristem) (Kerk *et al*. 2000). Also radish root segments exposed to exogenous IAA have shown the inhibition of tip growth and the increase of lateral root frequency with local increase in ascorbate oxidase which in turn is responsible for local depletion of reduced ascorbic acid (Kerk *et al*. 2000). Thus for the IL7-3 an auxin stimulation of lateral root growth could be responsible of the increased total-AsA content with concomitant decrease of AsA percentage. In any case, other hormones could have caused the observed alterations in ILs, for example a decrease of auxin/cytokinin ratio could increase the development of lateral buds (Salisbury *et al*. 1994) as it was observed in IL7-3.

4. Conclusion

The investigated introgression lines IL12-4 and IL10-1 of *S. pennellii* into *S. lycopersicum* genome have confirmed the already know higher and lower ascorbic acid accumulation in fruit, respectively, as compared to the cropped M82 genotype, whilst it is the first time that IL7-3 appeared to be an ascorbic acid overproducer. Although the ascorbic acid distribution in vegetative tissues of all investigated genotype was in agreement with that usually expected in herbaceous plants, for IL7-3 the higher total-AsA and the lower percentage of AsA on total-AsA in roots than the other genotypes, and the different morphological traits (shorter and ramified roots, higher number of lateral shoots and thick leaves, than M82) suggested a modification of IL7-3 metabolism. Such modification is probably due to reduced invertase activity and/or different hormones regulation/distribution in IL7-3 plant. This may have also caused the increase of ascorbic acid in its fruit. Again, at our knowledge, no gene involved in ascorbic acid biosynthesis and recycling is reported until now to be introgressed from *S.*

pennellii in IL7-3 genome as well as in IL10-1 and IL12-4 (Stevens *et al*. 2007). However, the total-AsA increase observed in IL7-3 fruits may depend by their lower water content that have to be investigated.

Further, yield and quality of crop of all ILs should be ascertained to asses the commercial and health value of fruits. In this context, IL7-3 and IL12-4 seem good candidates because they show better properties in terms of fruit sugar and ascorbic acid contents. In addition for IL7-3 higher resistance to biotic stress, and probably also to water stress, is expected because the ascertained presence of resistance gene I-3 from *S. pennellii* towards *Fusarium Oxysporum* (Hemming *et al*. 2004).

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Table 1. Total ascorbic acid (tot-AsA), expressed as mg/100g of FW, and relative percentage of reduced ascorbic acid (AsA%) in fruits of *S. lycopersicum vr* M82 and ILs

Genotype	**M82**	**IL7-3**	**IL10-1**	**IL12-4**
Tot-AsA	21.5 ± 1.5 c	40.6 ± 5.7 a	16.9 ± 1.3 c	28.6 ± 1.4 b
AsA%	77.6 ± 4.1 a	87.6 ± 3.7 a	77.1 ± 4.7 a	81.2 ± 3.3 a

Table 2. Morphological analysis of *S. lycopersicum* vr M82 and IL7-3 plants

	M82	**IL7-3**
Nodes (n)	15.0 ± 1.0 a	15.0 ± 0.6 a
Lateral shoots (n)	4.5 ± 0.4 b	8.0 ± 0.6 a
Leaves (n)	21.0 ± 1.8 b	28.0 ± 1.1 a
Leaflet area (cm^2)	40.4 ± 0.5 a	23.3 ± 1.2 b
Leaf area (cm^2)	150.7 ± 13.3 a	159.6 ± 22.9 a
Leaf DW (mg)	579.3 ± 139.0 a	321.3 ± 29.2 b
Petiole DW (mg)	163.4 ± 32.8 a	197.7 ± 6.5 a
Roots (n)	59.0 ± 15.0 a	80.0 ± 11.0 a
Root length (cm)	41.5 ± 3.9 a	32.7 ± 3.2 b
Aerial DW (g)	7.0 ± 1.8 a	6.8 ± 0.9 a
Roots DW (g)	1.2 ± 0.2 a	1.3 ± 0.1 a

Plant height (cm)	52.2 ± 2.2 a	42.0 ± 1.1 b

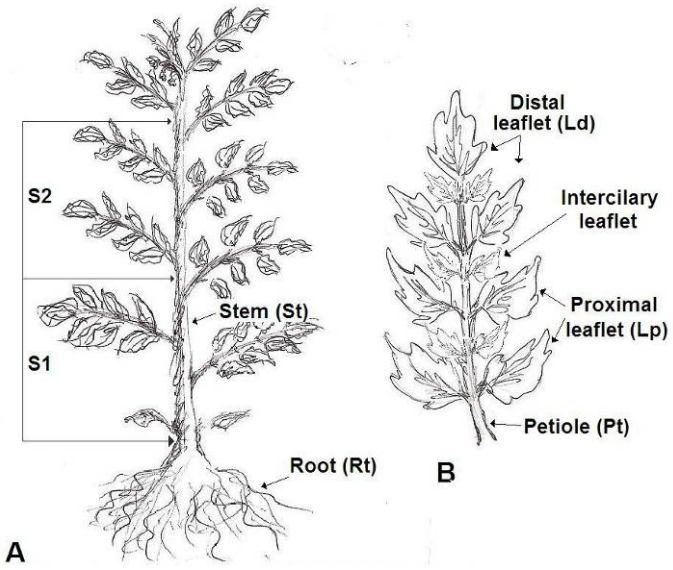

Figure 1. Tomato plant (A) and leaf (B). S1 and S2 are the sections from which samples were collected

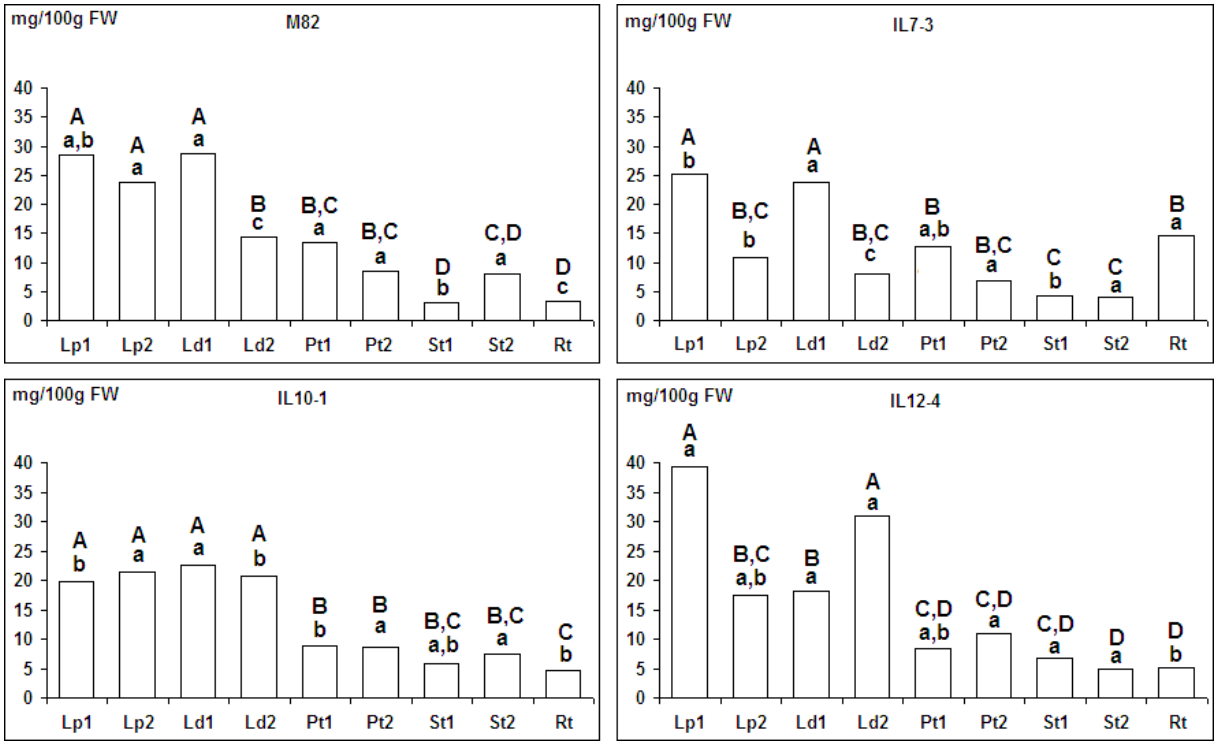

Figure 2. Total ascorbic acid content in leaf, petiole, stem and root of *S. lycopersicum* vr M82 and ILs, expressed as mg/100g of FW. Lp1, Ld1, Pt1 and St1 are proximal leaflets, distal leaflets, petiole and stem from section 1, respectively; Lp2, Ld2, Pt2 and St2 are from section 2, respectively; Rt, roots. Uppercase and lowercase letters represent statistically significant differences ($p \leq 0.05$) among tissues and genotypes, respectively

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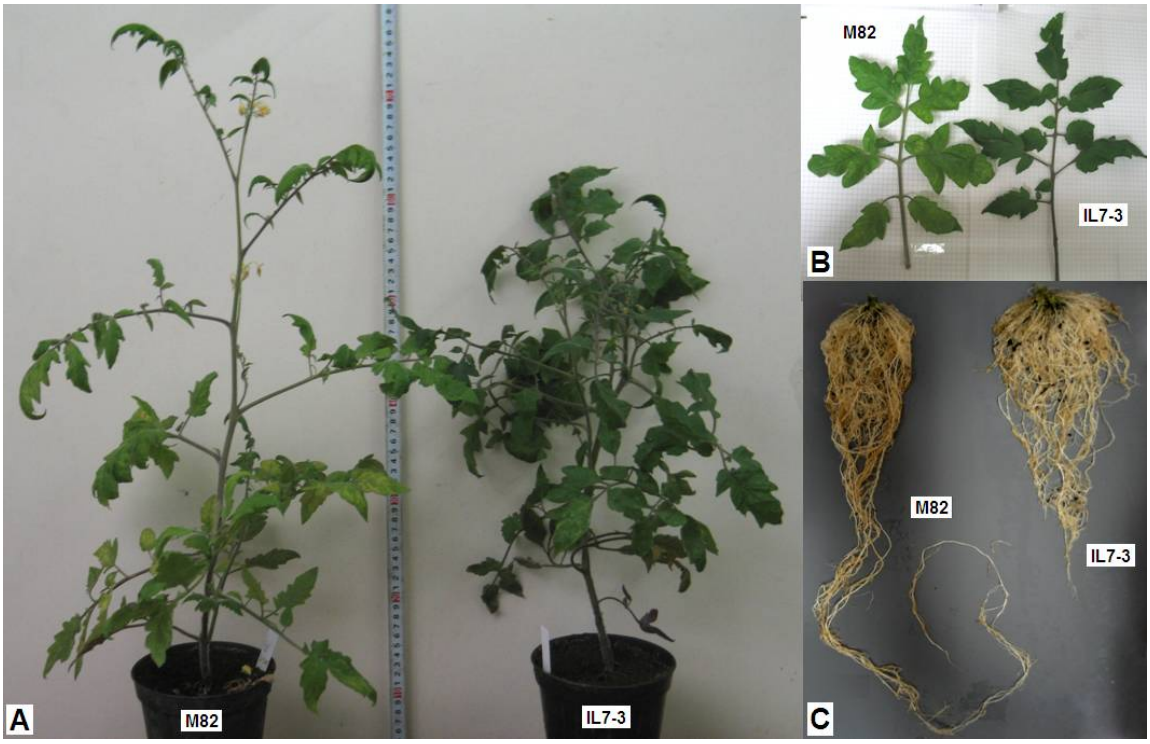

Figure 3. IL7-3 and M82 plants (A), leaves (B) and roots (C)

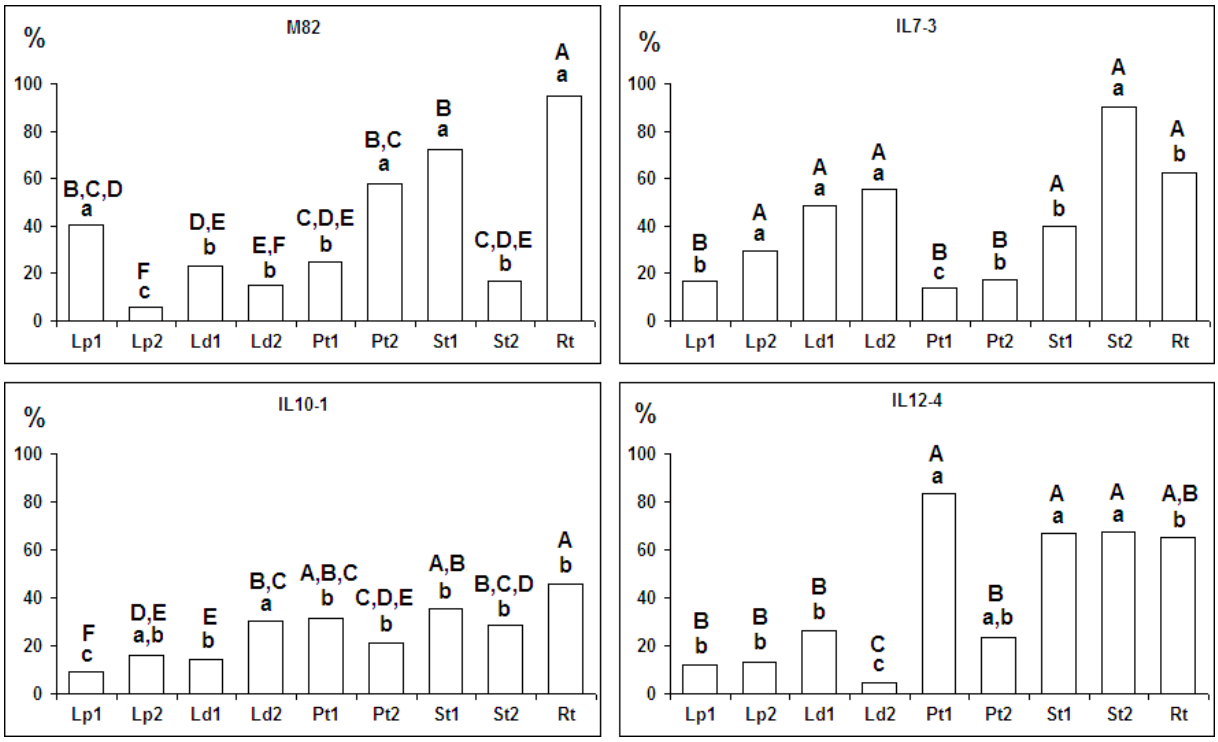

Figure 4. Percentage of AsA on total-AsA in different organs of tomato plants. Uppercase and lowercase letters represent statistically significant differences ($p \leq 0.05$) among tissues and genotypes respectively. Lp1, Ld1, Pt1 and St1 are proximal leaflets, distal leaflets, petiole and stem from section 1, respectively; Lp2, Ld2, Pt2 and St2 are from section 2, respectively; Rt, roots