Title	Overexpression of the acerola (Malpighia glabra) monodehydroascorbate reductase gene in transgenic tobacco plants results in increased ascorbate levels and enhanced tolerance to salt stress
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Relation	

Title:

Overexpression of the acerola (*Malpighia glabra*) monodehydroascorbate reductase gene in transgenic tobacco plants results in increased ascorbate levels and enhanced tolerance to salt stress.

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Abstract

Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) is a key enzyme of the ascorbate (AsA)-glutathione cycle that maintains reduced pools of AsA and serves as an important antioxidative enzyme. Previously, we have cloned MDHAR cDNA from acerola (*Malpighia glabra*), a plant that accumulates abundant amount of AsA**.** In this study, MDHAR cDNA from acerola was introduced into tobacco plants using an *Agrobacterium*-mediated gene delivery system. Transgenic tobacco plants accumulated greater amounts of AsA and showed higher MDHAR activity than the control plants. Lipid peroxidation and chlorophyll degradation, which were stimulated in control plants, were restrained in transgenic plants subjected to salt stress. These results indicate that overexpression of acerola MDHAR imparts greater tolerance to salt stress.

Keywords:

Acerola (*Malpighia glabra*); Ascorbic acid; Monodehydroascorbate reductase; Salt stress; Transgenic.

Abbreviations:

AsA, ascorbic acid; DHA, dehydroascorbate; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; *MgMDHAR*, *Malpighia glabra* MDHAR; NOS, nopaline synthase; ROS, reactive oxygen species; T-AsA, total ascorbic acid.

1. Introduction

Unable to move from their natural environment, higher plants undergo many unfavorable conditions such as drought, salinity, and extreme temperatures. Salinity is a major environmental factor leading to the deterioration of agricultural land and reduction in crop productivity (Vaidyanathan et al., 2003). About one-third of the world's cultivated land is estimated to be affected by salinity (Kaya et al., 2002). In plants, salinity causes diverse adverse effects such as the production of reactive oxygen species (ROS). These interact with a number of cellular molecules and metabolites, thereby leading to various destructive processes and cellular damage (Ashraf, 2009). ROS can seriously damage chlorophyll, proteins, membrane lipids, and nucleic acids (Alscher et al., 1997).

Plants possess an antioxidant defense system, which protects them from ROS. This system includes ascorbate (AsA), glutathione and α-tocopherol in addition to antioxidative enzymes such as catalase, superoxide dismutase, peroxidases, and enzymes involved in the AsA-glutathione cycle. Among the antioxidants, AsA plays a central role in defense against oxidative stress (Smirnoff, 1996). We previously reported an increase in AsA levels and tolerance to oxidative stress in transgenic tobacco cells expressing L-galactono-1,4-lactone dehydrogenase (Tokunaga et al., 2005). The AsA-glutathione cycle includes enzymes such as ascorbate peroxidase, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase and glutathione reductase. Ascorbate peroxidase simultaneously catalyses the reduction of H_2O_2 and the oxidation of AsA with the generation of monodehydroascorbate (MDHA). MDHAR may be critical in maintaining proper AsA concentrations by directly reducing MDHA to AsA (Asada, 1999). The active AsA-glutathione cycle plays an important role in the efficient removal of excess ROS (Noctor and Foyer, 1998; Asada, 2006). Protective functions provided by AsA and related antioxidant enzymes against photooxidative stress in chloroplasts have also been reported (Noctor and Foyer, 1998). Many antioxidant genes have been engineered in plants to tolerate oxidative stress. Transgenic potato plants over expressing L-gulono-γ-lactone gene isolated from rat were found to withstand various abiotic stresses caused by methyl viologen, NaCl or mannitol (Hemavathi et al., 2010). Recently Li et al. (2010) reported that overexpression of

chloroplastic MDHAR enhanced tolerance to temperature and methyl viologen-mediated oxidative stresses in tomato.

Acerola (*Malpighi*a *glabra*) is a tropical plant that accumulates large concentrations of AsA. We showed previously that acerola exhibits a much greater expression of AsA-biosynthetic enzymes than the model plant *Arabidopsis thaliana* (Badejo et al., 2009a). We also cloned the acerola AsA-recycling gene *MgMDHAR* and showed that it is vital for maintaining a high ascorbate redox status during stress conditions (Eltelib et al., 2011). Here we successfully generated transgenic tobacco plants overexpressing *MgMDHAR* and evaluated the performance of transgenic plants under salt stress conditions.

2. Materials and methods

2.1. Plant materials, plasmid construction and plant transformation

Tobacco plants (*Nicotiana tabacum* L.) were grown on MS (Murashige and Skoog, 1962) medium and maintained in a growth chamber at 25 °C under a 16/8-h light/dark photoperiod. The cDNA encoding *MgMDHAR* (accession number AB558587) was inserted into the *Bam*HI and *Sst*I sites of the binary vector pBI121 (Clontech, CA, USA). This replaced the *β*-glucuronidase reporter gene downstream of the CaMV 35S promoter and upstream of the NOS terminator to construct the expression vector pBI-*MgMDHAR* (Fig. 1A). This construct was delivered into *Agrobacterium tumefaciens* strain LBA4404 by electroporation and then into tobacco using the leaf disc method described previously (Badejo et al., 2008), resulting in *MgMDHAR*-transformed tobacco. T_0 transgenic plants were allowed to self-pollinate and T_1 seeds were collected and germinated on MS medium containing 100 μg mL^{-1} kanamycin. Non-transformed plants seeds were germinated on antibiotic-free MS medium. Transgenic and non-transformed plants were maintained at 25 °C under a 16/8-h light/dark photoperiod. Seven weeks later, the seedlings were transplanted into soil and maintained at 25 °C.

2.2. DNA isolation, PCR, and DNA blot analysis

Genomic DNA was extracted from tobacco leaves by the CTAB method described earlier (Eltelib et al., 2011). To detect the transgene, we performed PCR analysis using the sets of gene specific primers (5′-GGCTATTCGGCTATGACTGGGCAC-3′), and (5′-ATCACGGGTAGCCAACGCTATGTCC-3′) for the kanamycin resistance gene (*nptII*) and (5′-ATGGCAGAGAAGACTTTCA-3′) and (5′-GATCTTACAGGCAAA GGAGAGG-3′) for the *MgMDHAR* gene. The amplified DNA fragments corresponding to *nptII* (628 bp) and *MgMDHAR* (1304 bp) were detected by electrophoresis in 1% agarose gels. We performed DNA blot analysis as described previously (Eltelib et al., 2011).

2.3. RNA isolation and RNA blot analysis

We confirmed the expression of *MgMDHAR* in the transgenic lines by RNA blot analysis (Eltelib et al., 2011). Total RNA was isolated from tobacco leaves as described previously (Badejo et al., 2009b), and subjected to RNA blot analysis.

2.4. Salt stress treatment

Three independent transgenic lines (AM12, AM15, and AM18) and control tobacco plants were subjected to salt stress treatment. Seedlings were irrigated daily with Hyponex nutrient solution (HYPONeX, Osaka, Japan). Thirty five days after transplanting, three replications were used in salt stress evaluation experiment. The average plant height and number of leaves per plant for both control and transgenic plants were about 12 cm and 7 leaves per plant, respectively, before the application of salt treatment. The plants were subjected to salt stress treatment by applying 300 mM NaCl solution supplied with 1 mL L^{-1} Hyponex solution. This was renewed everyday for 9 days. The plants were grown under a light intensity of about 150 μmol m^{-2} s^{-1} and a 16/8-h light/dark photoperiod at 25 °C.

2.5. MDHAR enzyme assay and ascorbate determination

The enzyme activity of MDHAR, total ascorbate (T-AsA) as well as reduced ascorbate (AsA) were determined as described earlier (Eltelib et al., 2011). Dehydroascorbate (DHA) was calculated as the difference between T-AsA and AsA.

2.6. Determination of chlorophyll content

The chlorophyll content of the tobacco leaves was determined according to the method of Porra et al. (1989). Leaves were homogenized in 1.0 mL of *N,N'*-dimethylformamide and centrifuged at 20,000 × *g* for 10 min at 4°C. The supernatant was collected and its absorbance was measured at 665 and 647 nm. Three plants of each line were used for sampling. For both control and transgenic plants, the third leaf from the top was used for sampling.

2.7. Lipid peroxidation assay

Malondialdehyde (MDA) content was determined as previously described by Dionisio-Sese and Tobita (1998). Tobacco leaves were harvested and ground in liquid nitrogen. Finely ground leaves (0.1 g) were homogenized in 2 mL of 50 mM potassium phosphate buffer (pH 7.0) and centrifuged at 20,000 × *g* for 30 min at 4 °C. The resulting supernatant was then mixed with the same volume of 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) trichloroacetic acid. The mixture was heated at 95 °C for 30 min and the reaction stopped by placing it in an ice bath. The ice-cooled mixture was then centrifuged at 10,000 × *g* for 10 min and the absorbance recorded at 532 and 600 nm. After subtracting the nonspecific absorbance at 600 nm, the MDA concentration was calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$. Three plants of each line were used for sampling. For both control and transgenic plants, the third leaf from the top was used for sampling.

2.8. Statistical analysis

Three replicates of each sample were used for statistical analysis. Data were analyzed with Student's *t*-test; means were considered statistically significant at $P<0.05$.

3. Results

3.1. Generation of transgenic tobacco plants overexpressing the MgMDHAR gene

In this study we transformed tobacco plants with pBI-*MgMDHAR* (Fig. 1A) using an *Agrobacterium*-mediated gene-delivery system. Transgenic tobacco plants were obtained on MS medium containing kanamycin (T_0 plants). The T_0 lines were then transplanted to soil for cultivation. We found no significant phenotypic difference between the control and transgenic plants. T_1 seeds were obtained from all *MgMDHAR*-transgenic plants by self-pollination. T-DNA integration in the transgenic lines (AM12, AM15 and AM18) was confirmed by PCR detection of *nptII* and *MgMDHAR* genes. Both genes were present in the three transformants but none was found in the control (Fig. 1B and C). We used DNA blot analysis with *MgMDHAR* cDNA as a probe to verify the presence of *MgMDHAR* and estimate the copy-number of the transgene integrated into the isolated genomic DNA of the controls and transgenic plants. We found a positive hybridization signal only in the transgenic plants (Fig. 1D). The estimated copy-number of the *MgMDHAR* gene inserted into different transgenic lines ranged from one to two. RNA blot analysis indicated that *MgMDHAR* mRNA was expressed in the transgenic plants, but not the control plants (Fig. 1E).

Transgenic lines AM12, AM15, and AM18 exhibited a significant increase in MDHAR activity up to 1.8-, 1.6- and 2.1-fold, respectively, when compared to control plants (Fig. 2A). We next set out to determine whether transgenic tobacco lines expressing *MgMDHAR* showed increased levels of AsA. The AsA contents of the three lines were approximately 1.6 to 2 times higher than those of the control plants (Fig. 2B). The increase in the reduced ascorbate content produced an increase redox state (AsA/DHA) in the transgenic lines (Fig. 2B). The proportion of reduced ascorbate was about 63% in the control plants, while it was 83%, 81%, and 84% in the AM12, AM15, and AM18 lines, respectively.

3.2. Effect of salt stress on AsA content, chlorophyll content and lipid peroxidation

The AsA contents of the transgenic lines continued to be significantly higher than that of the control plants during salt stress treatment (Fig. 3A). Throughout the salt stress period, the AsA/DHA ratio of the transgenic lines was approximately 2.5- folds higher than the AsA/DHA ratio of control plants (Fig. 3B).

Exposure to salt stress resulted in a reduction in the chlorophyll content of both control and transgenic plants (Fig. 3C). Interestingly, the chlorophyll content of the transgenic lines was significantly higher than that of the control plants over the entire salt stress period. Six days after the initiation of salt treatment, the chlorophyll content of the transgenic lines AM12, AM15, AM18, and control plants decreased by 51%, 53%, 49%, and 74 %, respectively.

Lipid peroxidation measured as MDA content, a product of lipid peroxidation, is considered an indicator of oxidative damage (Dhindsa and Mathowe, 1981). MDA increased dramatically under salt stress in the control plants. The transgenic lines also showed an increase in MDA content (Fig. 3D). However, the MDA content of transgenic lines was significantly lower than that of the control plants throughout the stress treatment period (Fig. 3D). This indicates that the extent of lipid peroxidation in the control plants was significantly higher under salt stress. After 9 days of salt stress treatment, the MDA contents of the transgenic lines AM12, AM15, and AM18 showed an increase of 69%, 65%, and 48%, respectively, compared with 110% for the control plants.

4. Discussion

Plants growing naturally are often prone to different environmental stresses, including salinity, which is a major agricultural problem in arid and semi-arid regions (Ashraf and Akram, 2009). Previously, we showed that *MgMDHAR* is vital for maintaining a high ascorbate redox status during salt stress in acerola plants (Eltelib et al., 2011). Here we examined the hypothesis that oxidative stress generated by high salt could be reduced in tobacco plants overexpressing *MgMDHAR*. We successfully generated transgenic tobacco plants by introducing *MgMDHAR* under the control of CaMV 35S (Fig. 1). The transgenic

tobacco overexpressing *MgMDHAR* gene showed 1.7- to 2.0-fold increase in AsA content (Fig 2B). Similar results were recorded by Eltayeb et al. (2007) who reported that transgenic plants overexpressing *Arabidopsis thaliana* MDHAR showed about 2-fold increase in AsA contents. Recently, Kavitha et al. (2010) reported that transgenic plants overexpressing chloroplastic MDHAR from the halophyte *Avicennia marina* exhibited about 1.5-fold increase in AsA contents. Transgenic tomato plants overexpressing tomato chloroplastic MDHAR showed about 1.2-fold increase in AsA levels (Li et al., 2010). The significantly higher AsA levels and AsA/DHA ratio in *MgMDHAR* transgenic plants may be due to the higher levels of MDHAR activity compared to control plants (Fig. 2A); they may also be responsible for the enhanced conversion of monodehydroascorbate to AsA.

Increasing AsA levels through overexpression of AsA related genes other than MDHAR has also been reported. Previously we reported that all transgenic tobacco plants overexpressing acerola AsA biosynthesis-related genes (GDP-D-mannose pyrophosphorylase, phosphomannomutase or GDP-L-galactose phosphorylase) showed 2- to 3-fold increases in AsA contents (Badejo et al., 2008; 2009b; 2009c). AsA contents of the transgenic tobacco plants expressing superoxide dismutase, ascorbate peroxidase and dehydroascorbate reductase genes in the chloroplasts were approximately 1.5-fold higher than that of the control plants (Lee et al., 2007). Chen et al. (2003) reported that the level of AsA increase up to 2.4-fold in the leaves of transgenic tobacco overexpressing Wheat DHAR.

Many cell compartments produce ROS. AsA which presents in the cytosol, chloroplast, vacuoles, mitochondria and extracellular matrix is involved in the protection of different cell compartments against oxidative damage. Chloroplastic and cytosolic AsA concentrations can be high (Asada, 1999). This situation probably reflects the high need in these compartments for scavenging of ROS (Potters et al., 2002). The ability of AsA to interact with various ROS demonstrates its modulating effect on plant tolerance to various stresses (Conklin and Barth, 2004). Before the salt stress treatment, MDA levels were similar in both control and transgenic plants. Despite the increase in MDA in both control and transgenic plants in response to salt stress, the transgenic plants accumulated

significantly less MDA throughout the stress period compared with control plants, suggesting that transgenic plants experienced less lipid peroxidation under salt stress. Our results are in line with the findings of Kavitha et al. (2010) who reported that transgenic tobacco plants overexpressing chloroplastic MDHAR from the halophyte *Avicennia marina* survived better under salt stress conditions compared to untransformed control plants. They also reported that salt stress caused a significant increase of about 100% in the lipid peroxidation in control plants, but no significant increase was observed in transgenic lines. Transgenic plants overexpressing tomato chloroplastic MDHAR showed lower level of lipid peroxidation under low and high temperature stresses. The level of TBARS an indicator for lipid peroxidation increased by about 65% in the transgenic lines and 80% in the control plants (Li et al., 2010). Higher MDHAR activity coupled to higher AsA contents and AsA/DHA ratio in the *MgMDHAR* transgenic plants likely provided greater tolerance to salt-mediated oxidative stress and may have directly reduced the rate of lipid peroxidation. The result also supports our previous report that transgenic tobacco cells expressing the L-galactono-1,4-lactone dehydrogenase gene produced more AsA and had greater resistance to oxidative stress (Tokunaga et al., 2005).

Components of AsA-GSH cycle are widely distributed in the cellular compartments where ROS scavenging is needed such as the chloroplasts, microbodies, mitochondria and cytosol (Ishikawa et al., 2005). Previous studies supported the physiological importance of the cytosolic forms of the antioxidants involved in the AsA-GSH cycle (Yabuta et al., 2004; Yoon et al., 2004). Lunde et al. (2006) suggested that cytosolic MDHAR is likely to play important roles in protecting the whole plant during abiotic stresses. Lower lipid peroxidation resulting from higher MDHAR activity under salt stress was also reported in tomato (Gautier et al., 2010). Furthermore, increased accumulation of AsA and upregulation of antioxidant enzymes were also shown to limit oxidative stress following excessive production of ROS (Davey et al., 2000). In our previous study the phylogenetic analysis suggested that *MgMDHAR* is classified as a cytosolic enzyme (Eltelib et al., 2011). Taken together our results with the above reports, we also suggest that the cytosolic MDHAR likely play a vital role in reducing membrane damage during salinity stress.

Under adverse circumstances, the chlorophyll level is a good indicator of photosynthesis activity. It has been found that salt stress is known to reduce the chlorophyll content of plants (Xu et al., 2008). To examine whether increased AsA content and MDHAR activity protect against salt-induced chlorophyll loss, we measured chlorophyll content in the control and transgenic plants during salt stress (Fig. 3C). Transgenic plants had greater chlorophyll levels during the salt stress period. Li et al. (2010) observed similar results and reported that the chlorophyll content of transgenic tomato plants overexpressing chloroplastic MDHAR was significantly higher than the chlorophyll content of the wild type under methyl viologen-mediated oxidative stress. Our results indicate that transgenic plants with a larger AsA pool, which may result from enhanced AsA recycling due to high MDHAR activity, are able to maintain high chlorophyll content under salt stress conditions. The elevated accumulation of the antioxidant MDHAR probably prevented the degradation of chlorophyll in the leaves. As a result, the high levels of chlorophyll in the transgenic lines could result in more efficient photosynthesis during salt stress.

In conclusion, we showed that transgenic tobacco plants overexpressing acerola MDHAR not only have a higher AsA content but also higher ascorbate redox status, reduced levels of lipid peroxidation, and maintained higher levels of chlorophyll under salt stress. Thus, we propose that overexpression of acerola MDHAR in transgenic tobacco plants provides protection from oxidative damage under salt stress conditions.

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Legends of figures

Figure 1.

Expression of *Malpighia glabra* MDHAR (*MgMDHAR*) in transgenic tobacco plants. **(A)** Schematic representation of *MgMDHAR* expression vector used to transform tobacco plants. CaMV 35S, cauliflower mosaic virus 35S promoter; GUS, β-glucuronidase reporter gene; Nos-ter, nopaline synthase terminator. **(B and C)** PCR using the kanamycin resistance gene *nptII* and *MgMDHAR* gene specific primers for detection of the transgene in the genome of the selected *MgMDHAR* transgenic lines. The size of the amplified products corresponds to the size of the cDNAs (*nptII*, 628 bp and *MgMDHAR*, 1304 bp). **(D)** DNA blot analysis for detection of *MgMDHAR* in the genome of *MgMDHAR*-transgenic lines. Genomic DNA (10 μg) from tobacco leaves were digested with *Eco*RI, fractionated on agarose gel, transferred to a nylon membrane and hybridized with digoxigenin-labeled cDNA probe of *MgMDHAR*. **(E)** RNA blot analysis for the mRNA expression levels of *MgMDHAR* in *MgMDHAR*-transgenic lines. Total RNA (10 μg) were extracted from tobacco leaves, and probed using *MgMDHAR* cDNA.

Figure 2.

Ascorbate contents and MDHAR enzyme activity of control (Ctrl) and *MgMDHAR*-transgenic lines (AM12, AM15 and AM18). **(A)** MDHAR activity. Data represent mean ± SE (n = 3). **(B)** AsA and DHA contents and the ratio of AsA to DHA. Data represent mean ± SE (n = 3).

Figure 3.

Effect of salt stress on control (Ctrl) and *MgMDHAR*-transgenic plants (AM12, AM15 and AM18). AsA contents **(A)** AsA/DHA ratio **(B)** Chlorophyll content **(C)** and malondialdehyde content **(D)** of the leaves of tobacco plants irrigated with a nutrient solution containing 300 mM NaCl for 3, 6 and 9 d. Data represent mean ± SE (n = 3).

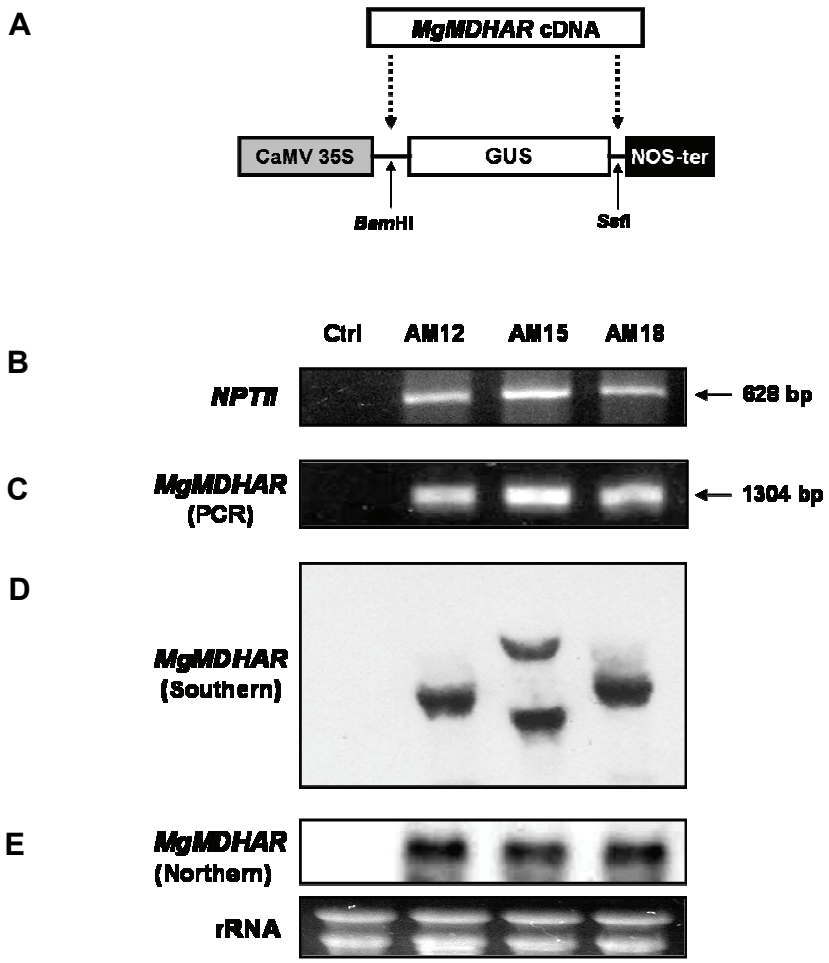

Figure 1. Eltelib et al.

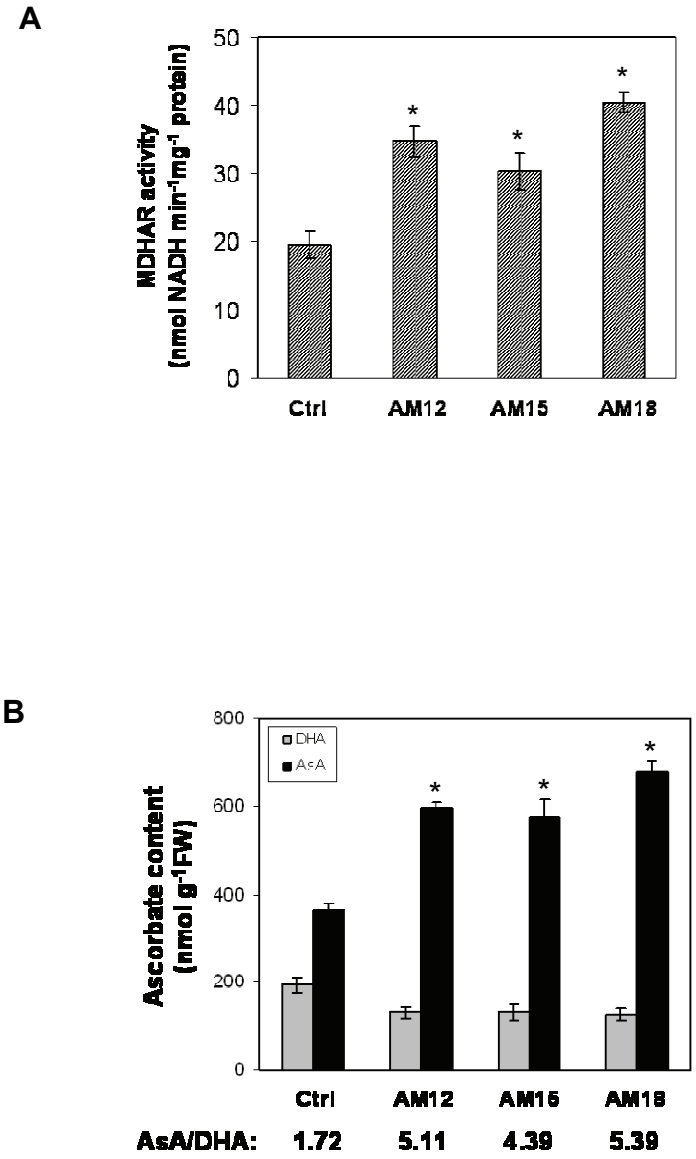

Figure 2. Eltelib et al.

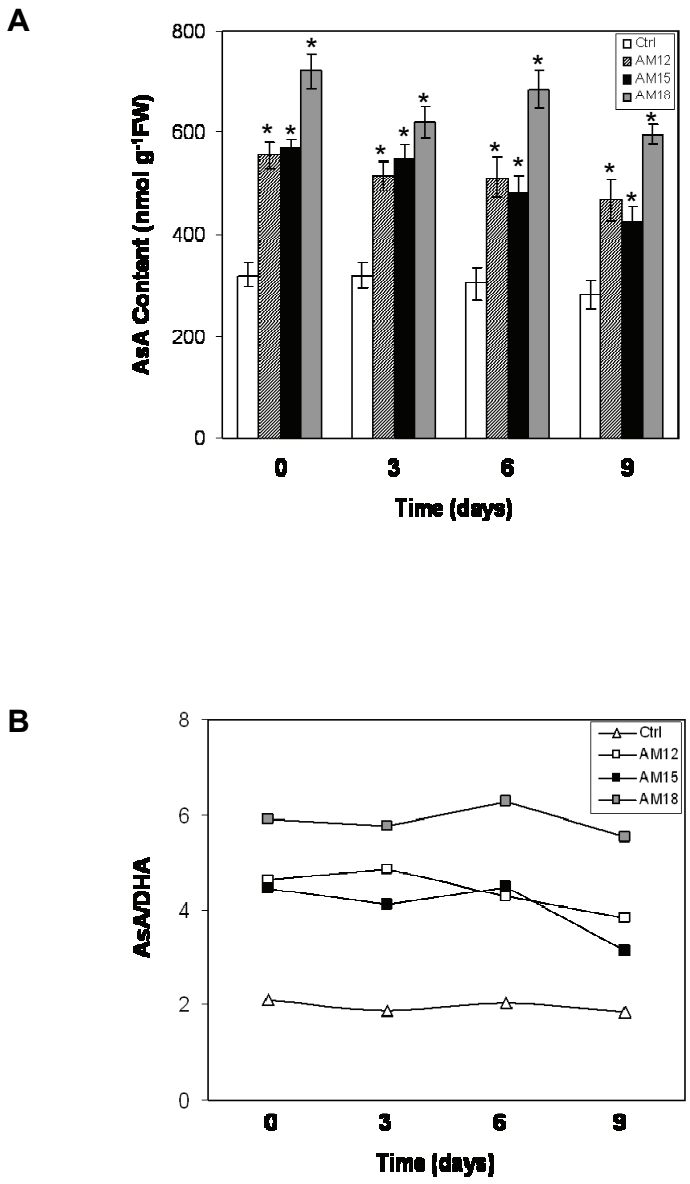

Figure 3. Eltelib et al.

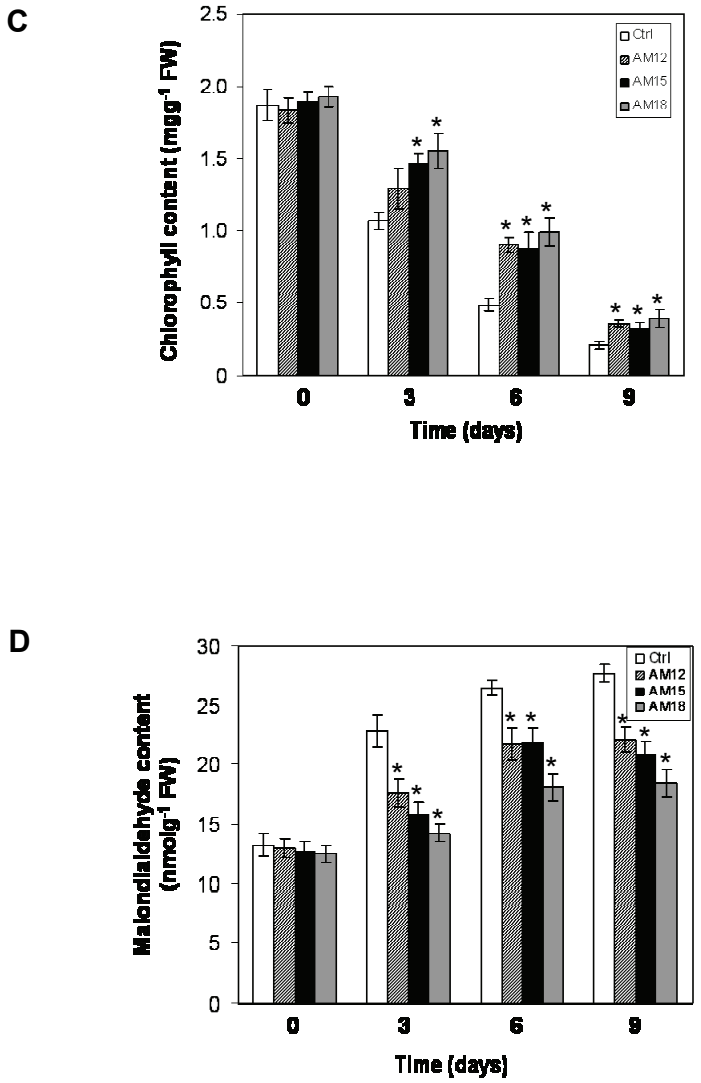

Figure 3. Eltelib et al.